TERMINATION OF EMBRYONIC DIAPAUSE IN THE RED KANGAROO, MEGALEIA RUFA, BY INJECTION OF PROGESTERONE OR OESTROGEN

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Summary. Injection of progesterone into lactating red kangaroos terminated embryonic diapause while the young continued to occupy the pouch. On Day 4, after progesterone injection on Days 1 to 3, the number of cells in the blastocysts was greater than during diapause and some glandular development had occurred in the uterus. By Day 8 the blastocysts had increased in size and the uteri were well developed.

When pouch young were removed (RPY) on Day 4 after injection of 10 mg progesterone/day on Days 1 to 3, the average time from RPY to birth in nine animals was shorter than in non-injected or in control-injected animals. The time from RPY to oestrus was similar in all groups. Not all animals gave birth earlier than expected, and at higher and lower dose levels the response was similarly heterogeneous. Two animals gave birth later than expected when treated with 20 mg/day for 3 days before RPY.

When pouch young were removed later than Day 4, birth occurred as early as 12 days after RPY.

Five of eleven animals gave birth after injection of oestradiol benzoate on Days 1 to 3 before RPY, and in three of the five the interval between RPY and birth was shorter than in non-injected animals.

INTRODUCTION

The red kangaroo, Megaleia rufa (Desmarest), usually comes into oestrus a day after giving birth; ovulation occurs about a day later and, if the female has mated, the egg is fertilized and develops into a unilaminar blastocyst containing about eighty-five cells (Sharman, 1963; Clark, 1966). The corpus luteum formed from the ruptured follicle also undergoes a short period of development and then, like the blastocyst, becomes quiescent without attaining its full size. While a young less than about 200 days old continues to occupy the pouch of the female, no growth or development occurs in either corpus luteum or

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blastocyst. Both corpus luteum and blastocyst resume development when the pouch young is approaching permanent emergence or if it is lost prematurely (Sharman, 1963). Several other macropodid marsupials, including the quokka, *Setonix brachyurus*, and the tammar, *Protemnodon eugenii*, follow a similar pattern of post-partum ovulation and embryonic diapause (Sharman, Calaby & Poole, 1966). In all marsupials that have been studied, histological observations show that the rate of growth and decline of the corpus luteum is independent of mating and pregnancy (Sharman, 1959; Tyndale-Biscoe, 1963a).

Secretions of the pituitary gland and of the ovaries are necessary for development of the blastocyst after removal of pouch young (RPy) in the quokka, but injection of progesterone into bilaterally ovariectomized animals permitted uterine and embryonic development after RPy (Tyndale-Biscoe, 1963a, b). The present study of the effect of progesterone or oestrogen on the uterus and blastocyst was made using intact red kangaroos in which the stimulus of the suckling young in the pouch maintained the quiescent phase of the corpus luteum.

**METHODS AND MATERIALS**

The animals used were drawn from the captive colony at the CSIRO Division of Wildlife Research, Canberra. They were kept in open yards and were maintained on a diet of natural pasture, lucerne and oats. A male was present with each group of females. All the females used in these experiments carried a pouch young of known age and were known to have mated at the post-partum oestrus.

Sterilized peanut oil, progesterone in synthetic oil (Fawns and McAllen, 10 mg/ml), or oestradiol benzoate in water (Oestroform aqueous, B.D.H.) was injected into the thigh muscles once daily (between 09.00 and 10.00 hours) for 3 days. The pouch young were not removed from some females; from others they were removed on the 4th, 11th or 18th day. After removal of the pouch young, the females were caught daily; the pouch was examined for the presence of a newborn young and the urogenital opening was checked for the presence of coagulated semen. Near the expected time of mating, a vaginal smear was taken every 2nd day, fixed in alcohol-ether, stained with Shorr’s stain and examined for evidence of oestrus and mating. Time intervals, e.g. between removal of pouch young and birth, were calculated by averaging the minimum and maximum time and adjusting to the nearest whole day; halves were taken to the higher whole number. The ovary bearing the corpus luteum of lactation and the corresponding uterus were removed by biopsy of animals anaesthetized with Nembutal (Abbott). The uterus was flushed with perfusion fluid (Cosol, CSL) and ovary and uterus were fixed for 48 hr in alcohol–formalin–acetic acid and stored in 80% alcohol. The blastocyst flushed from the uterus was measured with a micrometer eyepiece in a dissecting microscope before being fixed similarly.

Slices of the uteri and corpora lutea were embedded in wax, sectioned at 6 µ and stained with Ehrlich’s haematoxylin and eosin. Blastocysts were flattened and stained with Mayer’s haemalum by the method of Clark (1966).
RESULTS

Effect of injection of 10 mg progesterone for 3 days—histological observations

Five animals were injected with progesterone (10 mg) on Days 1 to 3. Two were laparotomized on Day 4, three on Day 8. The pouch young were not removed. Pouch young were removed on Day 1 from three non-injected control animals which were laparotomized on Day 1, Day 4 and Day 8, respectively.

Blastocysts

The blastocysts recovered from the two females on Day 4 had shell diameters of 330 µ, which is the same as the average shell diameter of the blastocyst in diapause. The diameters of the protoderm were 260 and 270 µ, respectively, compared with the average of 240 µ during diapause. However, these blastocysts consisted of 145 and 146 cells respectively, and several cells of each blastocyst were undergoing mitosis. The average number of cells during diapause is eighty-five and the cells are not known to divide (Clark, 1966). The protoderm of the two developing blastocysts had not differentiated.

Blastocysts were recovered from only two of the three females laparotomized on Day 8. These blastocysts had shell diameters of 420 and 690 µ, respectively, and both consisted of several hundred cells. Mitotic figures were numerous and differentiation of the protoderm had occurred.

The blastocyst recovered from the control animal on Day 1 had a shell diameter of 290 µ, protoderm diameter of 250 µ, and consisted of 100 cells. A blastocyst was not recovered from the control animal laparotomized on Day 4. The blastocyst recovered on Day 8 had a shell diameter of 290 µ, protoderm diameter of 240 µ, and consisted of eighty-six cells. In none of these characteristics could it be distinguished from a blastocyst in diapause. However, a single nucleus was seen to be dividing, and suggested that development had begun.

Ovaries and uteri

The corpus luteum of post-partum ovulation during the quiescent phase in the red kangaroo is a round body about 3 mm in diameter (Sharman, 1964b). The luteal cells are small and fairly densely packed; the nuclei are small and round, with chromatin condensed on the nuclear membrane and none of them is dividing (Pl. 1, Fig. 1). Blood vessels are not prominent. The corpora lutea from animals on Day 4 or Day 8 after progesterone treatment could not be distinguished from typical quiescent corpora lutea (Pl. 1, Fig. 3). On Day 4 after removal of pouch young (RPY) the corpus luteum was similar, but on Day 8 the luteal cells were larger and the intercellular spaces were also larger (Pl. 1, Fig. 5). Mitotic figures were seen in both luteal and connective tissue cells, but they were not frequent. Sharman (1964a) found mitoses in luteal cells 8 days after RPY.

The microscopic appearance of the uterus during the quiescent phase has been described by Newsome (1964). The large uterine glands are plentiful, well-coiled and evenly scattered throughout the endometrium. The epithelial cells lining the lumen and glands are columnar; their nuclei are elongate and situated near the base of the cells (Pl. 1, Fig. 2). The appearance of the uterus
on Day 4 after RPY was similar to the uterus in the quiescent phase, but on Day 4 after progesterone treatment changes had occurred. The glands were not larger or more numerous than during diapause, but the cell nuclei were no longer regularly basal. In one animal, the uterine stromal cells had expanded considerably so that the glands were widely spaced. Stromal expansion was not apparent in the other animal. On Day 8 after progesterone injection, the uteri were fully developed (Pl. 1, Fig. 4). Blood vessels were prominent in the stroma, and, in transverse sections, glands were more numerous than during the quiescent phase. The gland cells were tall, with vacuolated cytoplasm and small, round nuclei situated almost at the bases of the cells and forming a regular layer around the gland. The epithelium of the uterine lumen had become flattened and the cells were almost cuboidal, in contrast to their columnar shape during the quiescent phase.

At Day 8 after RPY a full luteal phase had not developed, although the stroma had expanded and the gland cell nuclei were not regularly basal (Pl. 1, Fig. 6). The appearance was similar to that of uteri on Day 4 after progesterone treatment.

Effect of injection of 10 mg progesterone for 3 days, followed by RPY

Microscopic examination (above) of blastocysts, recovered by hemi-hysterectomy of progesterone-treated animals, indicated that injection of progesterone terminated embryonic diapause in four animals, despite the presence of the suckling young. As hemi-hysterectomized animals were unsuitable for further experiments, most animals were not laparatomized and the pregnancies proceeded to term; a significant shortening of the time between RPY and parturition indicated that the treatment given before RPY had terminated embryonic diapause.

Ten animals were injected on Days 1 to 3 with progesterone (10 mg) or with peanut oil (1 ml). Pouch young were removed on Day 4. The results are shown in Table 1.

A paired $t$-test on the decrease in time from RPY to birth after progesterone treatment (the time from RPY to birth for each animal after peanut oil injection being used as the standard) indicated that the probability of the observed decrease being due to chance was less than 2%. The time between RPY and birth in progesterone-injected animals ranged from 25 to 33 days, and the variance was significantly different at the 1% level from the variance of control-injected animals, in which the range was from 31 to 34 days.

A paired $t$-test on the interval between RPY and oestrus in control-injected and hormone-injected animals failed to indicate significant difference at the

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EXPLANATION OF PLATE 1

Fig. 1. Corpus luteum of red kangaroo during embryonic diapause. × 410.
Fig. 2. Transverse section (t.s.) of uterus during embryonic diapause. Uterine lumen at lower right. × 170.
Fig. 3. Corpus luteum on Day 8 after injection of progesterone on Days 1 to 3. Pouch young not removed. × 410.
Fig. 4. t.s. uterus of same animal as Fig. 3. Uterine lumen at upper right. × 170.
Fig. 5. Corpus luteum on Day 8 after removal of pouch young on Day 1. × 410.
Fig. 6. t.s. uterus of same animal as Fig. 5. Uterine lumen at upper left. × 170.
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5% level, and the variances of the mean times in the two classes were not significantly different at the 1% level, although they were different at the 5% level.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Mean interval (days) and standard error</th>
<th>RPY to birth</th>
<th>RPY to oestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not injected*</td>
<td>8</td>
<td>31.3 ± 0.35</td>
<td>34.0 ± 0.89</td>
<td></td>
</tr>
<tr>
<td>Control-injected</td>
<td>8</td>
<td>32.4 ± 0.33</td>
<td>33.1 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>Progesterone-injected</td>
<td>10</td>
<td>28.3 ± 0.80</td>
<td>33.6 ± 0.80</td>
<td></td>
</tr>
</tbody>
</table>

*Data from Sharman (1963).

Effect of different doses of progesterone

When progesterone was given at the rate of 10 mg/day for 3 days, the time between RPY and birth varied widely among animals, and this variation suggested that 10 mg/day may not have been the optimum dose. Eight animals were injected daily with 5 mg and nine with 20 mg progesterone; as in the previous section, the animals were injected on Days 1 to 3 and the pouch young were removed on Day 4. The results are shown in Table 2.

**Table 2**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time from RPY to birth (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peanut oil 1 ml/day</td>
</tr>
<tr>
<td>K13</td>
<td>−, 31</td>
</tr>
<tr>
<td>K28</td>
<td>Not tested</td>
</tr>
<tr>
<td>K32</td>
<td>33</td>
</tr>
<tr>
<td>K36</td>
<td>Not tested</td>
</tr>
<tr>
<td>K58</td>
<td>32</td>
</tr>
<tr>
<td>K60</td>
<td>33</td>
</tr>
<tr>
<td>K62</td>
<td>34</td>
</tr>
<tr>
<td>K85</td>
<td>32</td>
</tr>
<tr>
<td>K96</td>
<td>32</td>
</tr>
<tr>
<td>K171</td>
<td>32</td>
</tr>
<tr>
<td>Mean</td>
<td>32.4</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.3</td>
</tr>
</tbody>
</table>

−, No birth.

* Neonatus survived less than 2 days.

† Blood released through urogenital opening 32 days after RPY.

Seven progesterone-treated animals gave birth 27 days or less after RPY at one or more dose levels. One animal gave birth only 23 days after RPY, i.e. 27 days after the first progesterone injection.
Three animals failed to give birth sooner than 30 days after RPY at any dose level given, and, of these, two gave birth 35 days after RPY when treated with the highest dose (20 mg/day).

Twelve times in thirty-three trials (36%) progesterone-treated animals failed to give birth, whereas only once in nine trials (11%) did a control-injected animal fail to give birth. In non-injected animals, about 85% of post-partum matings gave rise to a neonatus after RPY (Sharman, 1964a).

**Table 3**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Day of RPT</th>
<th>Time from RPY to birth (days)</th>
<th>Time from RPY to oestrus (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K60</td>
<td>11</td>
<td>19*</td>
<td>35</td>
</tr>
<tr>
<td>K171</td>
<td>11</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td>K32</td>
<td>18</td>
<td>12*</td>
<td>34</td>
</tr>
<tr>
<td>K58</td>
<td>18</td>
<td>33</td>
<td>33</td>
</tr>
</tbody>
</table>

* Neonatus survived less than 2 days.

**RPY on Day 11 or Day 18**

Four animals which had given birth less than 30 days after RPY when injected with 10 mg/day progesterone were again treated for 3 days with 10 mg/day, but the young were not removed till Day 11 (two animals) or Day 18 (two animals). At least 14 days elapsed between birth resulting from progesterone treatment and first injection of the following treatment. The results are summarized in Table 3.

**Table 4**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dose oestrogen (µg/day)</th>
<th>Time from RPY to birth (days)</th>
<th>Time from RPY to oestrus (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K113</td>
<td>20</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>K103</td>
<td>60</td>
<td>25*</td>
<td>34</td>
</tr>
<tr>
<td>K344</td>
<td>60</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>K86</td>
<td>100</td>
<td>24*</td>
<td>34</td>
</tr>
<tr>
<td>K96</td>
<td>100</td>
<td>30</td>
<td>32</td>
</tr>
</tbody>
</table>

* Neonatus survived less than 2 days.

At the time of death of the new-born young of K60 and K32, milk could not be manually expressed from the teat. A new-born young transferred to the pouch of K32, 31 days after RPY, died on the teat a day later. Apparently the mammary glands of K32 failed to produce milk in response to the stimulus of a young on the teat, both at the time of parturition and also at the time after RPY when birth would have been expected in a non-treated animal.
Effect of injection of oestrogen

Oestradiol benzoate (20, 60, 100 or 200 µg/day) was injected into eleven animals on Days 1 to 3 and the pouch young were removed on Day 4. The times between RPV and birth in the five animals which subsequently gave birth are shown in Table 4.

One animal (K103) was laparotomized on Day 4 after 100 µg oestrogen/day for 3 days. The luteal cells of the corpus luteum resembled those of corpora lutea during the quiescent phase, but the corpus luteum of the oestrogen-treated animal was distinguished by an abundance of large blood vessels. The uterine glands did not differ from those seen during the quiescent phase; the uterine stroma had expanded, so that the glands were widely scattered, as in some progesterone-treated animals. The blastocyst was not enlarged, the outer diameter being 270 µ. It consisted of 186 cells, approximately ten of which were undergoing mitosis.

DISCUSSION

Progesterone injected into the lactating red kangaroo once daily for 3 days initiated the resumption of embryonic development in most animals. The stimulated blastocyst developed to a neonatus whether the corpus luteum was allowed to develop at the end of the injections or continued to be inhibited by the suckling stimulus for as long as 14 days. Similarly in another marsupial, the quokka, Tyndale-Biscoe (1963b) showed that progesterone permitted resumption of development of the blastocyst after RPV and simultaneous ovariectomy. The interval between the first progesterone injection and birth (28 to 30 days) was shorter than the normal interval between RPV and birth (31.34±0.89 days, Sharman, 1963). Similarly the uterus and blastocyst of a non-injected animal 7 days after RPV resembled those of animals examined only 3 days after the first progesterone injection. This difference (1 to 3 days) must represent the time lag between cessation of suckling and secretion of the physiological substance(s) stimulating uterine and embryonic development.

Sharman (1955) suggested that resumption of blastocyst development in the quokka depended on the appearance of a secretory endometrium. Tyndale-Biscoe (1963a) found that the quokka blastocyst resumed development simultaneously with the corpus luteum about 2 days after RPV, whereas the luteal phase did not develop in the uterus until Day 7. This suggested that the blastocyst might be stimulated directly by secretions of the corpus luteum rather than by the uterus. In the red kangaroo, 3 days after the first injection of progesterone, the number of cells in the blastocyst had increased and many cells were dividing. The corresponding endometrium had undergone some pregestational changes, including expansion of the stroma and changes in the positions of gland cell nuclei, but the glands had not developed. This is evidence in favour of a double action of progesterone, its effect being both directly on the blastocyst and also on the uterus.

Two animals that failed to show any response to daily doses of 5 or 10 mg progesterone gave birth 3 days later than expected when injected with 20 mg/day. In another experiment, one female treated with 10 mg/day for 6 days
before and 2 days after RPY gave birth 44 days after RPY (unpublished results). These observations indicate that in some conditions progesterone may actually inhibit resumption of development by the blastocyst.

In the red kangaroo, post-partum oestrus usually occurs within 4 days of parturition (34.00 ± 0.89 days after RPY, Sharman, 1963). The post-partum oestrus of progesterone-injected animals occurred at a similar time after RPY (33.1 ± 0.40 days), although parturition had occurred as long as 22 days previously. The difference between the developmental age of the corpus luteum and that of the embryo did not affect the growth and decline of the corpus luteum and subsequent maturation of a follicle. However, the low proportion of births in progesterone-treated animals as compared with control-injected or uninjected animals suggests that intra-uterine mortality occurred, and this may have resulted from the asynchrony of corpus luteum and uterus. Tyndale-Biscoe (1963b) found that embryos developed to term in bilaterally ovariectomized quokkas, but the ovaries were necessary for parturition.

Six neonates died soon after birth. They attached to a teat in the normal way, but died there a day or two later, and at that time neither milk, nor the clear fluid of the first day or two of lactation could be squeezed from the teat. These young were born between 12 and 25 days after removal of a previous young. The hormone balance in the mothers at the time of parturition was apparently unsuitable for mammary gland development and the stimulus of the neonatus on the teat failed to elicit milk secretion. In one female tested, the mammary glands responded to the suckling stimulus neither at birth nor 31 days after RPY.

Tyndale-Biscoe's demonstration that progesterone treatment permitted embryonic development in the quokka after ovariectomy at RPY, and the results of progesterone injection in the red kangaroo, suggest that progesterone is the physiological stimulus to embryonic development. However, in four of eleven animals oestradiol benzoate terminated diapause. The effect of injected oestrogen on embryonic diapause in the red kangaroo is being further investigated. As the progesterone-treated and oestrogen-treated animals were not ovariectomized, small amounts of endogenous oestrogen and progesterone would have been present. It is possible that oestrogen and progesterone act synergistically in the termination of embryonic diapause.

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REFERENCES

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