The development of rabbit eggs in the ligated oviduct and their viability after re-transfer to recipient rabbits

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SUMMARY

The development of fertilized rabbit eggs trapped in the oviduct by ligation was examined at 84, 96, 108 and 120 h p.c. in ten animals, five of which were superovulated. Out of 210 eggs recovered, only two failed to develop into early blastocysts. The proportion of blastocysts undergoing some expansion increased from 66% at 84 h to 100% from 96 h onwards, when a high proportion, 66–89%, had ruptured zonae pellucidae. The viability of the blastocysts was assessed by transfer to the uteri of recipient rabbits, in which the proportion developing to term fell from 62% at 84 h to 3% at 108 h.

A total of 643, 2- and 4-cell eggs recovered 30 h p.c. was transferred to the ligated oviducts of oestrous, early or mid-luteal does for periods of 24, 48 or 72 h. Development to the early blastocyst stage appeared to be faster in the luteal phase than in oestrous does. Of the eggs recovered after either 48 h or 72 h, 20–40% and 3–17% respectively were capable of developing to term. In general, the less advanced blastocysts survived better.

Three hundred and twenty-one 60 h morulae were transferred to the ligated oviducts of oestrous or mid-luteal does for 24, 48 or 60 h. Neither the rate of egg development nor viability was significantly affected by the endocrine status of the temporary recipient. The proportions of eggs developing to term after transfer to recipients were 50%, 20% and 0%.

It is concluded that the rabbit egg cannot develop beyond the early blastocyst stage in the oviduct, irrespective of the host’s endocrine status. If development is interrupted, the life-span of the blastocyst is very short. The rabbit blastocyst requires to undergo almost continuous development to differentiate normally.

INTRODUCTION

In mammals, with certain exceptions which include the cat (Manwell & Wickens, 1928), dog (Holst & Phemister, 1971), mink (Hansson, 1947) and pig (Pomeroy, 1955), the eggs enter the uterus about 3 days after ovulation, most commonly as morulae but sometimes as early blastocysts (see reviews by Boyd & Hamilton, 1952; Blandau, 1969). If fertilized eggs are restricted to the oviduct by ligation, they may develop to the early blastocyst stage in the mouse (Kirby, 1962; Orsini & McLaren, 1967; Weitlauf, 1971), rabbit (Pincus & Kirsch, 1936; Adams, 1958), rat (Alden, 1942) and sheep (Wintenberger-Torres, 1956), but apparently not in the pig where blastulation fails (Murray et al. 1971). Moreover,
the fertilized eggs of one species may survive and develop to the early blastocyst stage in the oviduct of another, e.g. the rabbit can act as host to: cow (Adams, Moor & Rowson, 1968; Lawson, Rowson & Adams, 1972), mouse (Brinster & Thomson TenBroeck, 1969), pig (Polge, Adams & Baker, 1972), and sheep (Lawson, Adams & Rowson, 1972). Presently, all available evidence suggests that irrespective of whether the egg is confined to a native or alien oviducal environment development fails at the early blastocyst stage and degeneration then occurs. Within species the loss of viability of tube-locked eggs has only been investigated in the sheep (Wintenberger-Torres, 1956) and mouse (Weitlauf, 1971). In the rabbit, Adams (1958) observed that though egg development failed at approximately 84 h p.c., there appeared to be some delay, estimated at 12 h, before signs of impending degeneration appeared. However, no direct estimate of the viability of such blastocysts is available, and since it is recognized that morphological criteria may be poorly correlated with developmental potential, the present work was undertaken. Another objective was to investigate the development and viability of either 2- or 4-cell eggs or late morulae following their transfer for limited periods to the oviducts of rabbits in different, well-defined endocrine states. The fate of 60 h morulae transferred to the oviduct on the 11th day of pseudopregnancy has been described recently (Adams, 1971).

MATERIALS AND METHODS

The animals are conveniently divided into three main groups according to treatment, consisting of (1) donors to provide fertilized eggs, (2) temporary recipients to which eggs were transferred for periods of 24–72 h, and (3) recipients in which the further development of these eggs was assessed. Three experiments were carried out. In Exp. 1 the native fertilized eggs were trapped in the oviduct by ligation for varying lengths of time before recovery and transfer. In Exp. 2 and 3 eggs recovered either 30 or 60 h p.c. were transferred to the oviducts of temporary recipients, which were either oestrous, early luteal or mid-luteal. Altogether a total of 232 sexually mature does was used; 38 in Exp. 1, including 10 donors and 28 recipients; 120 in Exp. 2, including 26 donors and 25 temporary recipients and 69 recipients; and 74 in Exp. 3, including 16 donors, 12 temporary recipients and 46 recipients. The experimental animals consisted mainly of a large breed (strain A) supplemented with Dutch belted stock (Adams, 1970a) derived from our own closed colonies.

Donors

All of the donors, except 5 in Exp. 1, were treated with a horse anterior pituitary preparation to induce superovulation, as described previously (Adams, 1971). Twelve hours after the final priming injection the does were mated with two fertile males and given 25 i.u. human chorionic gonadotrophin (Lutormone, Burroughs Wellcome) to ensure the induction of ovulation.
In Exp. 1 each of the donors' oviducts was ligated with cotton thread near the tubo-uterine junction at either 14–24 h p.c. or 36–48 h p.c. to prevent the eggs entering the uterus. Normally the rabbit egg enters the uterus between 72 and 80 h p.c. (Assheton, 1894; Gregory, 1930). Eggs were recovered from the donors at autopsy by flushing 2 ml warm (37 °C) sterile 0.9% NaCl solution through the excised oviducts, at 84–120 h p.c. (Exp. 1), 30 h p.c. (Exp. 2) or 60 h p.c. (Exp. 3). Using a zoom binocular microscope (×20 to ×80), the flushings were then examined for eggs which were counted and quickly classified according to stage of development and condition.

Temporary recipients and recipients

Does intended for use as temporary recipients were either left untreated (oestrous), or mated with a vasectomized male and, during the autumn and winter months, given an ovulating injection of HCG, either 24–30 h (early luteal) or 9–11 days (mid-luteal) before egg transfer. Recipients were similarly treated 48–96 h before egg transfer, which was arranged so that the stage of egg development, rather than the eggs’ absolute age, would be synchronous with the recipients’ luteal development. Thus, in Exp. 1 the recipients were 86–101 h p.c.; in Exp. 2 either 72 or 96 h p.c. for eggs that had spent 24–48 h or 72 h respectively in the temporary recipients; and in Exp. 3 either 72 h or 96 h for eggs that had spent 24 h or 48–72 h respectively in the temporary recipients. In one exceptional group (Exp. 2) involving re-transfer to the oviduct, the five recipients were 48 h p.c.

Laparotomy was performed under Nembutal (pentobarbitone sodium, 60 mg/ml Abbott Laboratories) and Fluothane (Halothane B.P., I.C.I. Ltd) delivered in oxygen. In the case of the temporary recipients, eggs were transferred to the oviducts using a smooth-ended pipette which was inserted via the fimbria to a depth of 3–4 cm before the fluid, about 0.02 ml, containing the eggs was gently expelled. Each oviduct was ligated near the utero-tubal junction with cotton thread. The subsequent recovery and examination of eggs from the temporary recipients were as described above. Apart from the small group already referred to, all re-transfers were to the uterine horns, using the technique described by Adams (1962). The recipients were kept till full term in order to record the number of young born. Pregnancy was diagnosed by palpation on the 11th day p.c.

RESULTS

Experiment 1: development of fertilized rabbit eggs trapped in the oviduct by ligation

The results of restricting fertilized eggs to the oviduct, expressed in terms of the stage of development attained and condition of the eggs at recovery, are shown in Table 1. At 84 h only early blastocysts were recovered, including one-third unexpanded and two-thirds expanding but still intact. Within the next 12 h the position changed significantly: thus, at 96 h all of the blastocysts had
Table 1. *Stage of development and condition of eggs recovered from the ligated oviducts of rabbits 84–120 h post coitum, and their survival after transfer to the uteri of synchronized recipients*

<table>
<thead>
<tr>
<th>Eggs recovered from ligated oviduct (h p.c.)</th>
<th>No. animals</th>
<th>No. eggs</th>
<th>Stage of egg development</th>
<th>Proportion of eggs developing to term (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Early blastocysts</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Un-expanded</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ruptured</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>3</td>
<td>41</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>96</td>
<td>4</td>
<td>76</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>108</td>
<td>2</td>
<td>65</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>1</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Expanded and in two-thirds of them the zona pellucida had ruptured. Thereafter, the proportion of blastocysts with ruptured zonae pellucidae increased still further, reaching 89% at 108 h, after which, however, there was no further change, except for progressive degeneration of the cellular component.

Practically all of these eggs were transferred to recipients. Of those recovered at 84 h a high proportion was capable of developing to term (62%), but by 96 h viability had declined to 35%, and it fell to almost zero during the next 12 h period. The declining pregnancy rate noted among recipients of 108 h eggs (3 out of 7 compared with 70% at 84 and 96 h) indicates that an increasing proportion of the older eggs was dying before or near the time of implantation.

Experiment 2: development of 2- and 4-cell eggs in the ligated oviduct of oestrous, early luteal or mid-luteal recipients

Altogether 643 eggs were transplanted to 9 oestrous, 9 early luteal and 7 mid-luteal recipients for periods of 24, 48 or 72 h. The results are presented in Table 2. The proportion of eggs recovered varied from 74.5 to 96.9%, with no consistent difference existing between groups. Irrespective of the hosts' endocrine status, all of the eggs recovered after 24 h were at the morula stage, containing approximately 16 cells, which is normal. At 48 h, however, development had proceeded further in the luteal phase animals, which yielded 60% unexpanded early blastocysts compared with only 21% in the oestrous recipients where the proportion of morulae was correspondingly greater. This slower rate of development was still apparent at 72 h as judged by the significantly higher proportion of morulae, namely 38% compared with 4%. At this time the two early luteal does yielded a high proportion of expanding early blastocysts (82%) whereas none of the blastocysts recovered from the mid-luteal phase doe had expanded. This variation may reflect inherent differences between donors rather than an effect of treatment.
Development and re-transfer of rabbit eggs

Table 2. The development of 30 h 2- and 4-cell rabbit eggs transferred to the ligated oviducts of oestrous, 1-day, or 9- to 11-day pseudopregnant rabbits

<table>
<thead>
<tr>
<th>Temporary recipient</th>
<th>Condition</th>
<th>No.</th>
<th>Time eggs spent in temporary recipient (h)</th>
<th>No. eggs Transferred</th>
<th>Recovered</th>
<th>Morulae</th>
<th>Expanded</th>
<th>Unexpanded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestrous</td>
<td>2</td>
<td>24</td>
<td>55</td>
<td>41</td>
<td>41</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Early luteal</td>
<td>3</td>
<td>24</td>
<td>65</td>
<td>63</td>
<td>63</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mid-luteal</td>
<td>2</td>
<td>24</td>
<td>55</td>
<td>49</td>
<td>49</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oestrous</td>
<td>5</td>
<td>24</td>
<td>142</td>
<td>116</td>
<td>92</td>
<td>24</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Early luteal</td>
<td>4</td>
<td>48</td>
<td>85</td>
<td>75</td>
<td>50</td>
<td>45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mid-luteal</td>
<td>4</td>
<td>48</td>
<td>118</td>
<td>101</td>
<td>39</td>
<td>62</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oestrous</td>
<td>2</td>
<td>72</td>
<td>36</td>
<td>34</td>
<td>13</td>
<td>16</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Early luteal</td>
<td>2</td>
<td>72</td>
<td>60</td>
<td>49</td>
<td>2</td>
<td>7</td>
<td>40</td>
<td>—</td>
</tr>
<tr>
<td>Mid-luteal</td>
<td>1</td>
<td>72</td>
<td>27</td>
<td>24</td>
<td>1</td>
<td>23</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 3. The survival of 30 h 2- and 4-cell rabbit eggs exposed for 1–3 days to the ligated oviducts of oestrous, 1 day or 9- to 11-day pseudopregnant recipients, and then re-transferred to the uteri of synchronized recipients

(In parentheses the no. of recipients pregnant on day 11.)

<table>
<thead>
<tr>
<th>Eggs in temporary recipient</th>
<th>Condition of temporary recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Recips.</td>
</tr>
<tr>
<td>——</td>
<td>——</td>
</tr>
<tr>
<td>24</td>
<td>5 (3)</td>
</tr>
<tr>
<td>48</td>
<td>12 (11)</td>
</tr>
<tr>
<td>72</td>
<td>4 (2)</td>
</tr>
</tbody>
</table>

* Tubal transfer

Details of the various transfer groups are given in Table 3. No more than 10% of the morulae recovered after spending 24 h in the temporary recipients subsequently developed to term after transfer to the recipients' uteri and the results were no better following transfer to the oviduct. The relatively low pregnancy rate indicates that some pregnancies failed completely either before or about the time of implantation. Of the eggs that spent either 48 h or 72 h in temporary recipients, 20–40% and 3–17% respectively subsequently developed to term. The lowest rate of survival involved the early luteal group which contained
Table 4. The development of 60 h morulae transferred to the ligated oviducts of oestrous or 9- to 11-day pseudopregnant rabbits, and their survival after transfer to the uteri of synchronized recipients

<table>
<thead>
<tr>
<th>Temporary recipient</th>
<th>Time eggs spent in temporary recipient (h)</th>
<th>No. eggs Transferred</th>
<th>Re-</th>
<th>Recovered</th>
<th>Morulae</th>
<th>Early blastocysts</th>
<th>Un-</th>
<th>Expanding</th>
<th>No. young born/no. eggs trans-</th>
<th>Condition</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestrous</td>
<td>24</td>
<td>40 37 1</td>
<td>29</td>
<td>7</td>
<td>20/37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>145 40 4</td>
<td>30</td>
<td>6</td>
<td>22/39</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid-luteal</td>
<td>48</td>
<td>198 83 3</td>
<td>22</td>
<td>58</td>
<td>16/80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>192 71 15</td>
<td>18</td>
<td>38</td>
<td>13/71</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestrous</td>
<td>60</td>
<td>16 16 0</td>
<td>4</td>
<td>12</td>
<td>0/24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid-luteal</td>
<td></td>
<td>130 24 1</td>
<td>5</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a significantly higher proportion of expanded early blastocysts than either the ‘oestrous’ or ‘mid-luteal’ groups. Once again the low pregnancy rate suggests that the majority of the blastocysts succumbed very early, probably prior to implantation.

Experiment 3: development of 60 h morulae in the ligated oviduct of oestrous and mid-luteal recipients

A total of 321, 60 h morulae was transferred to the ligated oviducts of six oestrous and six mid-luteal does, where they were allowed to remain for 24, 48 or 60 h. The results are presented in Table 4. The proportion of eggs recovered, which varied from 77 to 100 %, appeared to be little affected either by the endocrine status of, or time spent in, the temporary recipients. Equally it appears from Table 4 that egg development was relatively unaffected, except that its rate was somewhat slower in the luteal than in the oestrous does. After 48 h, for example, 21 % of the eggs transferred to luteal phase does were still morulae compared with only 3-6 % in the oestrous does, and there was a corresponding difference in the proportion of expanding early blastocysts (53-7 % versus 69-9 %). The development of the eggs recovered after 60 h was similar irrespective of the endocrine status of the temporary recipient.

Practically all of the eggs were re-transferred to synchronized recipients where their viability proved to be almost identical, irrespective of previous treatment. Of eggs that had spent 24 h in a temporary recipient more than 50 % developed to term, but after a further 24 h only 20 % survived, with a decline to zero after a further 12 h. The sharp decline in viability is reflected in the pregnancy rate, which fell from 92 % (12/13) to 56 % (13/23) to 0 % (0/4) for the 24, 48 and 60 h groups respectively. In the 48 h group a high proportion of the eggs must have
died either before or near the time of implantation, and this applies particularly
to those from the oestrous temporary recipients.

Representative examples of eggs recovered from animals in Exp. 1, 2 and 3 are
shown in Fig. 1 (C–H), which also includes two normal early blastocyst stages
(A, B) for comparative purposes. The results relating to the viability of the eggs
shown in Tables 1, 3, 4 are depicted diagrammatically in Fig. 2.

**DISCUSSION**

The present results confirm earlier findings (Pincus & Kirsch, 1936;
Adams, 1958) that rabbit eggs fail to develop beyond the early blastocyst stage
when restricted to the oviduct. They also show that up to 96 h p.c. a substantial
proportion of such early blastocysts was capable of developing to term, but
that during the next 12 h viability was rapidly lost. A similar situation was found
to exist when 60 h morulae were cultured *in vitro* for 1–4 days (Adams, 1970b).
In that case, too, blastocyst expansion was limited and rupture of the zona
pellucida was common. In the rabbit the action of a special uterine protein,
‘blastokinin’, whose appearance coincides with cavitation and blastocyst ex-
pansion has been invoked as an inducer and regulator of blastocyst development
(Krishnan & Daniel, 1967). More recently it has been proposed that blastokinin
‘possibly acts at the transcriptional level in the role of a derepressor’ (Gulyas
& Krishnan, 1971). In an extensive review of the biochemistry of oviducal secre-
tions (Hamner & Fox, 1969) no mention was made of blastokinin having been
found in the rabbit oviduct, and recent work (Urzua, Stambaugh, Flickinger &
Mastroianni, 1970) indicates it is absent there. Feigelson & Kay (1972) consider
it improbable that a unique post albumin band, which they found in tubal fluid
of oestrous rabbits, is identical with blastokinin, though having similar electro-
phoretic properties.

In contrast to the fate of rabbit eggs transferred to the asynchronous uterus
(Adams, 1971), neither the rate of development of 60 h morulae in the ligated
oviduct nor their subsequent viability was significantly affected by the endocrine
status of the temporary recipient. Reference to Tables 1 and 4 shows that the
rate of development of the transferred eggs was slower than that of the native
eggs restricted to the oviduct; at 96 h p.c., for example, all native eggs had under-
gone expansion into early blastocysts, whereas even at 108 h (60 h morulae + 48 h),
30–46% of the transferred eggs still had not expanded. This retardation is
attributed to the effects of manipulation, as suggested earlier by Tarkowski
(1959) in the case of mouse eggs. It is notable that at equivalent times treatment
groups containing a higher proportion of the more advanced stages tended to
return lower rates of viability, as judged in terms of pregnancy rate and survival
to term; compare, for example, the performance of eggs subjected to ‘oestrous’
versus ‘early’ or ‘mid-luteal’ temporary recipients for 48 h (Tables 2, 3), or
108 h ‘native’ with 108 h ‘once transferred’ eggs (Tables 1, 4). One possibility is
Fig. 1.
that the less well developed eggs fortuitously enjoyed a more favourable uterine environment. However, this is unlikely because synchronization of the recipient was arranged so as to obviate or minimize any such effect. Rather it is postulated that the developing rabbit egg, having reached the early blastocyst stage, if deprived of some essential factor (whether blastokinin remains an open question) for even a short period will suffer irreparable harm. Previous observations from this laboratory indicate that the differentiating embryonic disc is most sensitive. When 'deprived' eggs are restored to a favourable environment (e.g. early luteal uterus) expansion of the zona pellucida and trophoblast proliferation may still occur though disc development fails, leading to the formation of trophoblastic vesicles (Adams, 19706). An initial failure of the zona pellucida to expand may
be a primary factor in abnormal disc differentiation, though equally such failure may be symptomatic rather than causal.

It appears that the rabbit blastocyst requires to undergo practically continuous development in order to differentiate properly, any discontinuity being poorly tolerated.

The finding that 2- and 4-cell eggs that had spent 48 h in a temporary recipient proved more viable than those that had spent only 24 h under the same conditions (see Table 3) reflects the greater degree of compatibility of more advanced morulae with the uterine environment. Though the total age of the 24 h group at re-transfer was about 54 h, the ‘retarding’ effect of two manipulations would render them even less compatible. In ‘straight’ transfers of 2-day eggs to either 2- or 3-day uteri only 20% developed to term (Chang, 1950), whereas 2½-day eggs were much more successful (Adams, 1962). The survival of eggs from the 48 h treatment group (Table 3), which ranged from 20% to 40%, was significantly lower than would be expected from ‘single’ transfers involving eggs of a similar age. The explanation could be that two series of manipulations actually have a deleterious effect on the egg, as postulated by Dickmann & De Feo (1967). Further, the present results suggest that certain cell stages may be more affected than others by such double manipulations.

The common occurrence of tubal ectopic pregnancy in man is exceptional; this condition is very rare in monkeys and apparently non-existent in domestic animals (Benirschke, 1969). Whether the human blastocyst is less demanding in its metabolic requirements or the oviduct more able to meet blastocyst demands compared with other species is not known. Progress in the culture in vitro of human embryos (Steptoe, Edwards & Purdy, 1971) should facilitate the investigation of these questions.

I am indebted to Mr M. L. Norris for assistance during the course of this work, especially with the anaesthesia.

REFERENCES


Development and re-transfer of rabbit eggs


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