Effect of post-ovulatory age of recipient on survival of frozen–thawed rabbit morulae

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Summary. A significantly higher survival rate was obtained when frozen–thawed morulae were transferred to oviducts of recipients ovulating after donors rather than to those of synchronized or earlier ovulating recipients. The highest proportion of live young, 23%, was obtained after transfer of eggs to recipients ovulating 18 h after donors. The proportion of live young (48%) was significantly increased when frozen–thawed rabbit morulae were transferred into both oviducts of recipients at this stage.

Introduction

Since the successful storage of mouse embryos at −196°C (Whittingham, Leibo & Mazur, 1972; Wilmut, 1972), the technique has been applied to the storage of rabbit embryos (Bank & Maurer, 1974; Whittingham & Adams, 1976; Maurer & Haseman, 1976; Tsunoda & Sugie, 1977a; Tsunoda, Soma & Sugie, 1978b; Yuhara, Nishimura & Utsumi, 1978; Tsunoda, Shimohora, Izumi, Soma & Sugie, 1979; Tsunoda, Soma & Sugie, 1981a). However, unlike the mouse, the viability of frozen–thawed rabbit eggs after transfer is usually low (but see Tsunoda & Sugie, 1977b; Parvex, Renard & Ozil, 1980) and is not correlated with their potential to develop in vitro.

Whittingham & Adams (1976) suggested that the low survival of frozen–thawed rabbit eggs after transfer might be due to (1) the damage to the zona pellucida and/or mucin coat during freezing and thawing and (2) unfavourable synchronization between egg and recipient. It is known that the degree of synchronization between donor and recipient is one of the important factors for a successful egg transfer in rabbits (Chang, 1950; Hafez, 1962; Fukumitsu & Sugie, 1974). There are various studies on the cryobiological factors which influence the survival of frozen–thawed rabbit eggs (Tsunoda et al., 1981b), but so far there is no report on donor–recipient synchrony in respect of the survival of frozen–thawed rabbit eggs.

Mullen & Carter (1973) reported that the transfer of unfrozen mouse eggs to both uterine horns of an individual recipient yielded a higher percentage of survival than transfer to only one uterine horn. So far, there is no report comparing the influence of unilateral or bilateral transfer on the survival of frozen–thawed eggs. The present study was undertaken to determine (1) the effect of post-ovulatory age of the recipient on the viability of frozen–thawed rabbit morulae and (2) whether pregnancy rate is influenced by transfer to one or both oviducts of an individual recipient.

Materials and Methods

Late morula-stage eggs were recovered in Ringer solution (0·147 m-NaCl, 0·004 m-KCl and 0·003 m-CaCl₂) containing 20% rabbit serum (Ringer + S) at 63 h post coitum from 61 mature
New Zealand White rabbits induced to superovulate with FSH and hCG as described by Tsunoda, Iritani & Nishikawa (1978a). The eggs were washed in two changes of medium and transferred to 0.1 ml PBS (Whittingham, 1971) supplemented with 50% rabbit serum (PBS + S). Dimethylsulphoxide was added three times at 37°C to give a final concentration of 1.5 m and the eggs were equilibrated for 15 min. The samples were placed in a chamber of an automatically controlled freezer (MP-1: MC-81, Tabai Manufacture Co., Japan) and cooled at 1°C/min from 20 to −5°C. The samples were induced to freeze at −5°C by local cooling of the wall with a pair of forceps previously cooled in liquid nitrogen. After 5 min, the eggs were cooled to −76°C at 1°C/min and transferred to liquid nitrogen vapour at −120°C for 5 min, and finally transferred to liquid nitrogen at −196°C and preserved for 2–79 days.

The samples were warmed from −70 to −5°C at approximately 4°C/min and the dimethylsulphoxide was diluted out in a step-wise manner by the addition of 0.8 ml PBS + S at 1-min intervals at 37°C as described elsewhere (Tsunoda et al., 1978b). After recovery, the eggs were washed twice in PBS + S, and the morphology of blastomeres and zonae pellucidae was examined. The eggs with intact blastomeres and zona pellucida were selected and stored in Ringer + S for up to 1 h before transfer.

The frozen–thawed eggs were transferred to the oviducts of 51 pseudopregnant does at different times after ovulation. Ovulation of recipients was stimulated to occur by the intravenous injection of 50 i.u. hCG 24 and 12 h before (−) or 0, 12, 18, 24 and 36 h after (+) ovulation in donors. The frozen–thawed eggs were transferred to one oviduct at different times after ovulation in the first experiment and transferred to both oviducts in the second experiment. The recipients were examined by laparotomy on Day 17 of pregnancy to determine the numbers and the condition of implanted embryos and then allowed to go to term. Statistical significance was determined by χ² tests.

**Results**

Of the 737 eggs frozen, 706 (96%) were recovered from the straws after thawing. Of the 706 eggs recovered, 339 (48%) had intact blastomeres and an undamaged zona pellucida.

In-vivo development of frozen–thawed rabbit morulae after transfer to recipients at different times after ovulation is summarized in Table 1. The highest pregnancy and implantation rates were obtained when the eggs were transferred to recipients induced to ovulate 18 h after ovulation in donors. The implantation rate in this group was significantly (P < 0.05 to

<table>
<thead>
<tr>
<th>Donor/recipient synchrony (h)</th>
<th>No. of recipients</th>
<th>No. pregnant (%)</th>
<th>No. eggs transferred</th>
<th>No. of implantations (%)</th>
<th>No. of live fetuses (%)</th>
<th>No. of live young (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−24</td>
<td>6</td>
<td>1 (17)</td>
<td>33</td>
<td>3 (9)**</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>−12</td>
<td>6</td>
<td>4 (67)</td>
<td>42</td>
<td>6 (14)**</td>
<td>5 (12)</td>
<td>5 (12)</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>2 (33)</td>
<td>38</td>
<td>5 (13)**</td>
<td>3 (8)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>+12</td>
<td>7</td>
<td>5 (71)</td>
<td>47</td>
<td>14 (30)</td>
<td>9 (19)</td>
<td>9 (19)</td>
</tr>
<tr>
<td>+18</td>
<td>6</td>
<td>6 (100)</td>
<td>31</td>
<td>12 (39)</td>
<td>7 (23)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>+24</td>
<td>6</td>
<td>3 (50)</td>
<td>46</td>
<td>11 (24)</td>
<td>6 (13)</td>
<td>6 (13)</td>
</tr>
<tr>
<td>+36</td>
<td>5</td>
<td>0</td>
<td>25</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

The injection of hCG in recipients was done to induce ovulation after (+) or before (−) that in donors. Significantly different from value at +18 h: * P < 0.05; ** P < 0.001.
Freezing of rabbit morulae

P < 0.001) higher than those obtained when the eggs were transferred to synchronized or older recipients, but was not different from those obtained after transfer to recipients ovulating 12 and 24 h after donors. The combined comparison of pregnancy groups showed that there was a significant difference (P < 0.05 to P < 0.01) between the proportion of females pregnant and numbers of implantations, live fetuses and live young after transfer to synchronized or older recipients and those obtained after transfer to young recipients, except at 36 h.

Table 2 shows the results following bilateral transfer of frozen–thawed rabbit morulae to recipients ovulating 18 h after donors. The proportion of live young after bilateral transfer was significantly (P < 0.05) higher than that after unilateral transfer (Table 1: 48 versus 23%). However, the proportion of implantations was not significantly different. Live fetuses were observed on both sides in 3 recipients but Rabbit 500, in which the eggs were transferred to the right uterus, had no live fetus on this side.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>No. of eggs transferred</th>
<th>No. of implantations (no. of live fetuses)</th>
<th>No. of live young</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>531</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>503</td>
<td>5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>502</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>500</td>
<td>3*</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Total (%)</td>
<td>33</td>
<td></td>
<td>6 (38)</td>
</tr>
</tbody>
</table>

* The eggs were transferred to the uterus because of bleeding from the oviduct during operation.

Discussion

In general, it is considered that the highest survival rates after egg transfer result from transfer to recipients that are synchronized with the donors or have ovulated slightly later (Chang & Pickworth, 1969). However, in the rabbit, high survival rates were obtained after transfer to recipients that had ovulated 12 h earlier than the donor (Adams, 1962; Fukumitsu & Sugie, 1974). Fukumitsu & Sugie (1974) briefly reported that a high proportion of live young (50–58%) was obtained when 3-day-old morulae were transferred to synchronized or older (6 and 12 h difference) uteri, but a low proportion of live young (33–38%) was obtained after transfer to younger (6 and 12 h difference) uteri. Although the site to which the embryos were transferred was different from that of the present study and the previous investigations in our laboratory (Fukumitsu & Sugie, 1974), it seems that frozen–thawed rabbit morulae should be transferred to younger recipients in comparison with unfrozen rabbit morulae. Freezing and thawing of eggs may delay the resumption of normal metabolic and synthetic activity in the thawed rabbit eggs as reported in the mouse by Whittingham (1975) and Whittingham & Anderson (1976).

The present study also demonstrated that a significantly higher proportion of live young was obtained after transfer of frozen–thawed rabbit morulae to both oviducts compared with transfer to only one oviduct. Mullen & Carter (1973) reported that the transfer of mouse eggs to both uteri yielded a high percentage developing and fewer resorptions than the transfer to one uterine horn. However, Adams (1962) found that pregnancy rate after transfer of rabbit eggs was not
influenced by whether the transfer was unilateral, and in mice an empty uterine horn does not exert a systemic or local luteolytic effect on the survival of eggs transferred to the other horn (McLaren, 1970). Because bilateral transfer of frozen–thawed rabbit morulae in the present study did not affect the proportion of implantations, the high proportion of live young observed after bilateral transfer was mainly due to prevention of post-implantation mortality, but the mechanisms are unclear. More eggs were transferred in the bilateral transfer experiment, but the effect of this difference was not investigated in the present study.

With our present freezing technique, 48% of the eggs transferred can be expected to develop into live young when morulae are frozen and thawed, and transferred to both oviducts of recipients ovulating 18 h later than the donors. This value is comparable with those obtained after transfer of unfrozen rabbit morulae (54%: Maurer, Hunt, Van Vleck & Foote, 1968; 50–58%: Fukumitsu & Sugie, 1974). Considering the proportion of eggs recovered from freezing straws, 96%, and the proportion of eggs which survive for transfer after thawing, 48%, 22% of the eggs originally frozen can develop into live young. We did not make an exact classification of the abnormalities in the thawed eggs which could not be used for transfer, but about half of them had intact blastomeres but a broken or ruptured zona pellucida. It is known that rabbit eggs without a zona pellucida fail to develop after transfer (Moore, Adams & Rowson, 1968). To improve the viability of frozen–thawed rabbit eggs, it seems to be necessary (1) to develop new freezing and thawing methods which can keep the zona pellucida intact and (2) to investigate possible variations of the transfer technique to produce full development of the eggs with broken or ruptured zonae after transfer.

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References


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