TRANSFER OF PORCINE EMBRYOS AFTER 3 DAYS OF IN VITRO CULTURE

Birgit Blum-Reckow and W. Holtz

University of Göttingen, Federal Republic of Germany

ABSTRACT

Two experiments were conducted to determine the viability of porcine embryos transferred after long-term in vitro culture. In Exp. 1, four-cell embryos were kept in culture for 120 h. Embryos that were exposed to fresh culture medium every 12 h survived better than embryos kept in the same medium throughout the culture period. In Exp. 2, four- and eight-cell embryos were cultured in vitro for 72 h before transfer to estrus-induced recipient gilts. Each gilt received, on average, 19 embryos. If recipients were synchronous with donors 3/32 (9%) recipients remained pregnant with an average of 4.0 ± .6 viable young. If the sexual cycle of the recipients was 24 h behind that of the donors the pregnancy rate was 18/34 (53%) with 4.4 ± .5 viable young. Average embryo survival rate for the two groups was 1.8 and 12.5%, respectively. A 24-hourly medium replacement during the in vitro culture period had no significant effect on transfer results. When transferring freshly collected blastocysts, pregnancy rate, number of viable young and survival rate of embryos were 6/10 (60%), 7.8 ± 1.4, and 23.9% for synchronous recipients and 7/10 (70%), 9.3 ± 1.8, and 32.9% for asynchronous recipients, respectively. Recipients with very high plasma progesterone levels or numerous follicular cysts at the time of transfer were less likely to remain pregnant than others.

Key Words: Embryo Transfer, Pigs, Synchronized Females, Culture Media, In Vitro Culture


Introduction

To date no suitable technique for freezing pig embryos has been reported. Temporary storage in a ligated rabbit oviduct has limited applicability (Polge et al., 1972; Herrmann and Holtz, 1985). Embryos destined for shipping over long distances must be kept in culture for several days. There are few reports on the transfer of porcine embryos cultured for 48 h or more, and these are based on small numbers of animals (Pope and Day, 1977; Davis and Day, 1978).

Materials and Methods

General Procedures. Prepuberal gilts (n = 149) and parous sows (n = 51) of German Landrace, German Yorkshire, Landrace × Yorkshire crosses, and Camborough hybrids served as embryo donors. At the time of treatment, gilts weighed 94 ± .7 kg, and sows weighed 172 ± 2.7 kg. To induce superovulation, donor gilts and sows received a single injection of 800 IU of pregnant mare’s serum gonadotropin (PMSG) and 400 IU of human
chorionic gonadotropin (hCG)\(^3\), followed by 500 IU of hCG\(^4\) 72 h later. Sows were treated 1 d after weaning following a 23- to 25-d suckling period. Gilts were inseminated 24 and 36 h after the hCG injection, using \(3 \times 10^9\) sperm from sires of proven fertility. The sperm were suspended in 100 ml of a commercial glucose-EDTA extender\(^5\). Sows were mated to a mature boar on d 2 and 3 after the hCG injection. Donors were slaughtered by \(500\) min after slaughter. The flushing medium was modified Dulbecco's PBS\(^6\) containing 1,000 mg/liter glucose, 36 mg/liter sodium pyruvate, 10,000 IU/liter penicillin, and 50 mg/liter streptomycin, with pH 7.2 and osmotic pressure 280 mosmol, supplemented with 1% heat-inactivated bovine serum. Flushing medium and all equipment were kept at 37°C. Immediately after recovery, embryos were inspected under the microscope and each time five donors were used. Embryos were considered normal if they had reached the four- to eight-cell stage and displayed symmetric blastomeres without signs of degeneration. Ten to 15 morphologically normal embryos were placed in covered sterile tissue culture dishes (34 × 10 mm)\(^7\) containing 3 ml of PBS supplemented with 20% heat-inactivated bovine serum. They were cultured in a water-jacketed incubator\(^8\) at 37 ± .5°C under moisture-saturated air.

Experiment 1: Effect of Replacement of Culture Medium on In Vitro Development of Four-Cell Embryos. In this experiment the effect of a 12-hourly replacement of culture medium on the in vitro performance of cultured four-cell embryos was investigated. The experiment was run in seven replicates, and each time five donors were used. Embryos were collected 72 ± 2 h after the ovulation-inducing hCG-injection. Four-cell embryos of different donors were pooled, randomly allotted to three treatment groups, and kept in culture for 120 h. Embryos of Group 1 were transferred to a dish with fresh medium every 12 h. Embryos of Group 2 were drawn into the pipette and redeposited into the original dish, simulating the act of transfer. Embryos of Group 3 remained untouched throughout the 120 h of culture. The appearance of the embryos was recorded at 24-h intervals by placing the covered culture dishes on the warmed stage of an inverted microscope (128 ×). Embryos were classified as four-cell stage, six- to eight-cell stage, morula, blastocyst, expanded blastocyst, hatching blastocyst, or hatched blastocyst. Embryos that did not continue to develop or showed signs of degeneration (disintegration, uneven blastomerses, etc.) were classified as degenerated or nonviable.

Experiment 2: Transfer of Embryos to Recipient Gilts after 72 Hours of In Vitro Culture. In this experiment the effect of an extended in vitro culture period on the transfer result was investigated. The experiment was run in 36 replicates. Each time between two and seven (\(X = 5\)) donors were superovulated.

Four- to eight-cell embryos were collected at 120 ± 2 h and blastocysts at 160 ± 2 h after the ovulation-inducing hCG injection. Morphologically normal four- to eight-cell embryos were randomly allotted to two groups. Embryos from Group 1 were transferred to dishes with fresh medium every 24 h, and those from Group 2 remained in the same medium throughout the culture period. After 72 h in culture, embryos of both groups were classified morphologically under the microscope at 128 ×. Embryos in the morula, blastocyst, expanding blastocyst, or hatching blastocyst stage with normal appearance were allocated randomly to recipients. Recipients were prepuberal German Landrace × German Yorkshire gilts with a mean age of 184 ± 1.5 d and weight of 96 ± .8 kg. They were estrus-induced by injection of 400 IU of PMSG and 200 IU of hCG, followed by 500 IU of hCG 72 h later. In half the recipients the treatment began simultaneously with that of the donors (synchronous group), whereas in the other half estrus was induced 24 h later (asynchronous group).

As a control, expanded blastocysts flushed from the uterus of donors slaughtered 160 ± 2 h after the hCG treatment were transferred to recipients within 2 to 3 h after collection (direct transfer).

---

3Double dose of "PG 600", Intervet, Boxmeer, Netherlands.
4"Ekluton", Intervet, Boxmeer, Netherlands.
5Merck, Darmstadt, FRG.
6Serva, Heidelberg, FRG.
7"Falcom", Beckton Dickinson, 6900 Heidelberg, FRG.
8Forma Scientific, Marietta, OH.
CULTURE OF PIG EMBRYOS

3337

Of the 86 recipients, 82 received between 15 and 25 embryos, two received 13, and one each 26 and 27 embryos (X = 19). Embryos transferred were in the expected stage of development and were morphologically intact. Slight imperfections, such as a few extruded blastomeres, were tolerated.

Recipients were anesthetized with azaperone\textsuperscript{9} and metomidate\textsuperscript{10} as described by Holtz (1987). A midline incision was made, ovaries were inspected, and embryos, suspended in .01 ml medium, were deposited in one uterine horn about 2 cm from the uterotubal junction. For that purpose embryos were aspirated into polypropylene tubing\textsuperscript{11} (i.d. .58 mm, o.d. .96 mm) attached to a 1-ml syringe. The uterine wall was punctured with a blunt hypodermic needle (i.d. 1.2 mm, o.d. 1.5 mm), the tube was threaded through the needle, and embryos were deposited in the lumen 1 to 2 cm from the tip of the needle. The abdominal incision was closed with three suture lines (abdominal wall, subcutaneous fascia, and skin).

Blood samples were collected from the marginal ear vein of 73 of the recipients at the time of surgery. After 6 to 10 h of storage at 4°C the serum was aspirated and frozen until it was analyzed for progesterone content by enzymeimmunoassay\textsuperscript{12}. Intra- and interassay CV were 13 and 13.5%, respectively, and the limit of detection was 1.3 pg/well. The applicability of the assay to progesterone determination in porcine serum has been established by Losert et al. (1986).

Recipients were slaughtered 28 to 36 d after the transfer, and fetuses were counted, weighed, and measured. Degenerate fetuses and fetuses with less than half the weight of the average normally develop ones were classified as nonviable.

In Exp. 1, differences among groups were tested for significance by a chi-square test. Experiment 2 used a two-way-classification design. Data were analyzed with Model 1 of the ANOVA program by Harvey (1976), with the fixed effects “exchange of medium” and “synchrony between donors and recipients” without interaction. When comparing pregnancy rates the chi-square test was applied.

Results

Experiment 1. As shown in Figure 1, the number of embryos developing progressively decreased with time in all groups. Within treatment groups each 24-h decrease was significant (P < .05). Replacement of medium at 12-h intervals reduced the rate of deterioration; the superiority of this group reached statistical significance after 72 h of culture (P < .05). After 96 and 120 h the difference became more pronounced (P < .01). Sham replacement of medium had no significant effect on the rate of deterioration of embryos (P > .05).

Experiment 2. Results of transfer of embryos to recipients after 72 h of culture are presented in Table 2. Of 32 recipients that were treated simultaneously with donors, only 3 (9%) remained pregnant, bearing an average of 4.0 live fetuses. This amounted to an average embryo survival rate of 1.8%. When recipients were treated 24 h later than donors, 18 out of 34 (53%) remained pregnant, yielding an average of 4.4 live fetuses. In this group, embryo survival rate averaged 12.5%. With 3 of 16 vs 0 of 16 recipients remaining pregnant with embryos from the no medium exchange group compared with the medium exchange group, respectively, a distinct effect of daily medium renewal was not evident.

When blastocysts were transferred within 2 to 3 h after collection (direct transfer) 6 of 10 recipients treated synchronously remained pregnant, yielding an average of 7.8 live fetuses. Corresponding data for recipients that were treated 24 h later than donors were 7 of

\textsuperscript{9}Stremil, Jannsen, Düsseldorf, FRG.
\textsuperscript{10}Hypnodil, Jannsen, Düsseldorf, FRG.
\textsuperscript{11}Hythe, Kent, UK.
\textsuperscript{12}Reproquant p, Noctech/Galway, Ireland.
TABLE 1. SURVIVAL RATE AND DEVELOPMENTAL STAGE REACHED BY LATE FOUR-CELL
AND EIGHT-CELL EMBRYOS CULTURED FOR 72 HOURS WITH OR WITHOUT
REPLACEMENT OF MEDIUM AT 24-HOUR INTERVALS (EXP. 2)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Medium renewal</th>
<th>No medium renewal</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of embryos cultured</td>
<td>822</td>
<td>871</td>
</tr>
<tr>
<td>Transferable embryos after 72 h, %</td>
<td>82.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.5</td>
</tr>
<tr>
<td>Morulae or early blastocysts, %</td>
<td>9.5</td>
<td>9.4</td>
</tr>
<tr>
<td>Blastocysts or expanded blastocysts, %</td>
<td>86.9</td>
<td>89.4</td>
</tr>
<tr>
<td>Hatching blastocysts, %</td>
<td>3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Different from the group without medium renewal (a = P < .01, b = P < .05, chi-square test).

10 pregnant and 9.3 live fetuses. This amounted to embryo survival rates of 23.9 and 32.9%, respectively.

The pregnancy rate of recipients with more than three ovarian follicular cysts > 10 mm in diameter was low (1/5) compared with animals with one to three cysts (14/34) or no cysts (19/47). Of the four nonpregnant recipients with more than three cysts, two each belonged to the synchronous and the asynchronous recipient group.

In Table 3, the pregnancy rate after transfer is presented in relation to serum progesterone level of recipients at the time of transfer. From 15 ng/ml upward pregnancy rates became progressively poorer. The difference reached a significant level when progesterone was higher than 20 ng/ml (P < .05).

Discussion

In this experiment we collected embryos in the late four-cell and early eight-cell stage by flushing donors 120 ± 2 h after the hCG injection, that is approximately 80 h after the expected time of ovulation (Dziuk and Baker, 1962; Hunter, 1972; von Kaufmann and Holtz, 1982). If embryos are collected earlier, the four-cell block, known to exist in the pig (Polge and Frederick, 1968; Rundell and Vincent, 1968; Wright, 1977; Davis and Day, 1978; Herrmann et al., 1981; Menino and Wright, 1982), hampers their development. If they are collected too late, embryos will hatch in vitro. Hatched porcine embryos are easily damaged and therefore are difficult to handle.

The culture medium used was Dulbecco's
TABLE 2. TRANSFER OF FOUR- TO EIGHT-CELL EMBRYOS CULTURED IN VITRO FOR 72 HOURS TO RECIPIENTS THAT WERE EITHER SYNCHRONOUS WITH OR 24 HOURS "BEHIND" DONORS. THE CULTURE MEDIUM WAS EITHER LEFT UNCHANGED OR RENEWED AT 24-HOUR INTERVALS. AS A CONTROL, FRESHLY COLLECTED BLASTOCYSTS WERE TRANSFERRED TO SYNCHRONOUS AND ASYNCHRONOUS RECIPIENTS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Synchronous recipients</th>
<th>Asynchronous recipients&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Direct transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No medium exchange</td>
<td>Medium exchange</td>
<td>Total</td>
</tr>
<tr>
<td>No. of recipients</td>
<td>16</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Avg no. of embryos transferred</td>
<td>(15 to 24)</td>
<td>(13 to 21)</td>
<td>(13 to 24)</td>
</tr>
<tr>
<td>Recipients pregnant&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>3 (19%)</td>
<td>0</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>Total fetuses per pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>× ± SE</td>
<td>4.0 ± .6</td>
<td>0</td>
<td>4.0 ± .6</td>
</tr>
<tr>
<td>Range</td>
<td>3 to 6</td>
<td>3 to 6</td>
<td>1 to 9</td>
</tr>
<tr>
<td>Viable fetuses per pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>× ± SE</td>
<td>4.0 ± .6</td>
<td>0</td>
<td>4.0 ± .6</td>
</tr>
<tr>
<td>Range</td>
<td>3 to 6</td>
<td>3 to 6</td>
<td>1 to 8</td>
</tr>
<tr>
<td>Embryo survival rate, %&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.0</td>
<td>0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Estrus was induced 24 h later than in donors.

<sup>b</sup>Upon slaughter 28 to 36 d after transfer.

<sup>c</sup>(Number of viable fetuses recovered from pregnant recipients/number of embryos transferred to all recipients) × 100.

<sup>d</sup>Different from corresponding synchronous recipients (P < .01, chi-square test).
TABLE 3. EFFECT OF SERUM PROGESTERONE CONCENTRATION OF RECIPIENTS AT THE TIME OF TRANSFER ON THE PREGNANCY RATE AFTER TRANSFER (EXP. 2)

<table>
<thead>
<tr>
<th>Serum progesterone, ng/ml</th>
<th>No. of recipients</th>
<th>Pregnant recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>5 - 10</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>11 - 15</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>16 - 20</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>21 - 30</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>31 - 60</td>
<td>15</td>
<td>1</td>
</tr>
</tbody>
</table>

*Different from the first four groups (P < .05, chi-square test).

PBS. It is particularly suited for shipping of embryos because it does not require gassing with CO₂ to maintain its pH. Our results (81% morphologically intact embryos after 3 d of culture) compare favorably with those reported by other groups working under comparable conditions with different media (Schneider et al., 1975; Pope and Day, 1977; Wright, 1977; Davis and Day, 1978; Lindner and Wright, 1978; Niemann et al., 1983; Stone et al., 1984). In this experiment all embryos were included unless they were unfertilized or degenerate. Better results may have been achieved by transferring only morphologically very good or excellent embryos.

A periodic replacement of culture medium had a favorable effect on embryo survival. No comparable investigation on porcine embryos was found in the literature, except a study by Kruff et al. (1984), the results of which do not confirm our findings. Because in this investigation the sham treatment (Exp. 1: drawing of embryos into a pipette and placing them back into the original medium) had no significant effect, the renewal of medium per se must have been beneficial. When shipping embryos over long distances, a frequent renewal of culture medium is impractical. Therefore, in our second experiment, the medium was replaced only once instead of twice daily. After 72 h of culture the advantage of medium replacement was evident, although less conspicuous than in Exp. 1. The explanation for the favorable effect of medium renewal is obscure. The amount of culture medium per embryo was large (10 to 15 embryos in 3 ml of medium), so it is unlikely that depletion of available substrates or accumulation of metabolites had reached critical stages. Nevertheless, it seems that culture in microdrops is, in all probability, not a suitable system for maintaining porcine embryos in culture for long periods of time. Pope and Day (1977) observed an embryo survival rate of only 8% after transfer of embryos cultured in microdrops for 48 h. In a direct comparison, Menino and Wright (1982) found significantly more cleavage divisions per unit time when culturing one-cell pig embryos in tubes compared with microdrops.

Thus far, there has been only one report on the transfer of porcine embryos that had been cultured for 3 d; Davis and Day (1978) attained two pregnancies when embryos were transferred to six recipients. The present investigation showed that reasonably good pregnancy rates may be attained, provided that the recipients are at an earlier stage of the cycle than the donors (18 out of 34 recipients pregnant with 4.4 viable fetuses). These results were not significantly different (P > .05) from those obtained by direct transfer of blastocysts (13 out of 20 recipients pregnant with 8.6 viable fetuses). In contrast, pregnancy rates with embryos cultured for 3 d were poor if estrous cycles of donors and recipients were synchronous (three out of 32 recipients pregnant with, on the average, four viable fetuses). The most likely explanation for the favorable effect of asynchrony between donor and recipient on pregnancy rates is that embryonic development in vitro proceeds at a reduced rate compared with development in vivo (Anderson, 1978; Lindner and Wright, 1978; Pope et al., 1982; Niemann et al., 1983) Similar observations have been reported for mice (Hahn, 1984) and sheep (Tervit and Rowson, 1974). A study by Wilde et al. (1988) indicated that blastocysts with delayed development will survive if transferred to a less-advanced uterine environment. The tolerance of porcine embryos with regard to the state of synchrony between donors and recipients (Webel et al., 1970; Polge, 1982) might apply to direct transfers, but it does not necessarily extend to embryos that have been in culture for a long period. The data presented here suggest that even freshly collected blastocysts survive better if the recipients are at an earlier stage of the cycle than the donors. It may be that manipulation of the embryos and short-term storage in vitro result in a temporary delay in embryonic development.
High plasma progesterone levels in recipients reduced pregnancy rates after embryo transfer. This contradicts reports on bovine embryos (Remsen and Roussel, 1982; Northey et al., 1985) showing that high progesterone levels favor successful transfer. At present one can only speculate on reasons and functional connections that may be involved. It seems to be advisable, though, to screen prospective recipients for their plasma progesterone concentrations before using them. Likewise, animals with more than two or three follicular cysts should be dismissed. Having analyzed blood samples for progesterone concentration only we have no plausible explanation for the unfavorable effect of the ovarian cysts. Possibly these problems are associated with the hormonal induction of estrus in recipient gilts.

When transferring porcine embryos, most researchers chose to work with early developmental stages. Only few investigators transferred morulae or blastocysts (Hunter et al., 1967; Webel et al., 1970; Pope et al., 1982; Pope et al., 1986; Stein-Stéfani and Holtz, 1987). None of these studies involved large numbers of recipients. In our earlier studies, when transferring four- and eight-cell embryos, average litter size in pregnant recipients was always around seven viable fetuses (Herrmann and Holtz, 1985; Schlieper and Holtz, 1986; Holtz et al., 1987; Holtz and Schlieper, 1991). In this and in an earlier study involving transfer of morulae and blastocysts (Stein-Stéfani and Holtz, 1987) the average litter size in pregnant recipients was 8.6 (n = 13) and 10.2 (n = 23), respectively. In all cases recipients were prepuberal gilts induced with PMSG and hCG. In a paper by James et al. (1983) higher pregnancy rates, but no difference in litter size, were reported when transferring morulae compared with four- and eight-cell embryos.

In summary, for the long-term culture of porcine embryos in PBS supplemented with glucose, sodium pyruvate, antibiotics, and 20% bovine serum, a periodic exchange of the medium is beneficial. When transferring embryos after 3 d in culture a pregnancy rate of approximately 50% may be attained, provided that the recipients are at an earlier stage of the estrous cycle than donors. Litter size, however, tends to be relatively small and variable. Unhatched blastocysts seem to be more suitable for transfer than embryos at earlier stages. Recipients with high plasma progesterone concentrations or with numerous follicular cysts have a reduced likelihood of remaining pregnant.

Implications

Reasonable pregnancy rates may be achieved when transferring porcine embryos after 3 d in vitro culture. Phosphate-buffered saline, supplemented with glucose, sodium pyruvate, antibiotics, and 20% bovine serum, requiring no gassing with CO₂, may serve as culture medium. A periodic exchange of culture medium may improve the survival rate of embryos in vitro. For transfers to be successful after 3 d of in vitro culture, recipients have to be at an earlier stage of the estrous cycle than donors. Transfer of freshly collected, unhatched blastocysts may lead to pregnancy rates of 60 to 70% with an average litter size of eight to nine viable young. Recipients with very high plasma progesterone levels or numerous follicular cysts are less likely to remain pregnant.

Literature Cited


