VIABILITY OF STORED EQUINE EMBRYOS


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ABSTRACT

Equine embryos were recovered nonsurgically 6.5 d after ovulation (Exp. 1) and those >200 μm were stored in one of three media: 1) Ham's F10 + 10% fetal calf serum (FCS) under 5% CO2, 5% O2 and 90% N2 at 24 C (Ham's F10); 2) Minimal Essential Medium with Hank's balanced salts + 10% FCS in air (MEM) at 24 C or 3) MEM at 5 C (n=10/treatment). Embryos ≤200 μm (n=10) were bisected microsurgically; one-half of each embryo was stored in Ham's F10 and the other half in either Dulbecco's phosphate-buffered saline + 10% FCS in air at 24 C (DPBS), or MEM in air at 24 C. At 0, 12 and 24 h, embryos were: 1) measured; 2) assigned a developmental score of 1 to 4 (1=tight morula, 4=expanding blastocyst) and 3) assigned a quality score of 1 to 5 (1=excellent, 5=degenerate). Whole embryos stored in MEM at 5 C or 24 C did not (P>.05) advance in development by 24 h, whereas those stored in Ham's F10 at 24 C were more (P<.05) advanced (i.e., higher developmental score) by 24 h. From 0 to 24 h, 1 of 10, 6 of 10 and 7 of 10 whole embryos developed when stored in MEM 5 C, MEM 24 C and Ham's F10 24 C, respectively. Embryo quality was better at 24 h (P<.05) for embryos stored in Ham's F10 at 24 C compared with MEM at 5 C. Quality of bisected embryos was better (P<.05) for those stored in Ham's F10 compared with MEM or DPBS. Experiment 2 evaluated the viability of embryos stored in Ham's F10 at 5, 24 or 37 C for 12 h and 37 C for an additional 12 h (n=10/treatment). Embryos cultured at 5 or 24 C did not (P<.05) develop or grow during the initial 12 h, but upon culture at 37 C they advanced (P<.05) in development. Experiment 3 was designed to determine pregnancy rates for embryos transferred nonsurgically after storage in Ham's F10 at 24 C for ≤1 h or 12 h. Treatments were: 1) embryos transferred ≤1 h into recipients that ovulated 1 d prior (+1) to 3 d after (-3) the donor mare; 2) transferred ≤1 h into progestin-treated recipients; 3) transferred 12 h into naturally synchronized recipients (+1 to -3 d in relation to donor) and 4) transferred 12 h into progestin-treated recipients. Progestin-treated recipients were administered .044 mg of alrtenogest/kg body weight daily beginning the day after the donor ovulated. Thirteen of 32 synchronized recipients became pregnant (d 30) compared with 0 of 17 for progestin-treated recipients (P<.05). Pregnancy rates for embryos stored for 12 h were nearly identical (P>.05) to those transferred in ≤1 h. Therefore, embryo viability was maintained for 12 h by storage in Ham's F10 at 24 C.

(Key Words: Embryos, Culture Media, Horses.)

Introduction

Limited data are available on methods for storage of equine embryos (Oguri and Tsutsumi, 1974; Allen and Rowson, 1975; Douglas, 1980). Culture systems that maintain viability of equine embryos for 12 to 24 h are essential for studies on sexing and bisecting embryos and for in vitro fertilization. Storage of embryos would be extremely advantageous. Embryos could be collected on one premise and transported to another facility for transfer. Short-term storage of equine embryos is more attractive than freezing because pregnancy rates are likely to be higher and most breed registries have not approved the use of frozen-thawed embryos.

Maintenance of recipient mares is a major cost of embryo transfer. Successful use of ovariecetomized progestin-treated mares as recipients (Hinrichs et al., 1985, 1986) offers many advantages. Apparently, no similar studies have been conducted using intact progestin-treated mares as recipients.

Oguri and Tsutsumi (1974) maintained 6-d-old embryos in saline + 2% gelatin or a mixture of mare's serum: Ringer solution (1:1) at 30 C for 29 to 70 min until transcervical transfer. There was no difference in the number of pregnancies due to storage in either media (4 of 10

1 Supported in part by Van Camp Foundation and Colorado State Univ. Exp. Sta. Alrtenogest was donated by Hoechst-Roussel-Agti Vet, Somerville, NJ. We thank Dr. Takeda for bisecting the embryos and assistance with culturing embryos.

Received November 20, 1986.
Accepted March 31, 1987.
and 2 of 5, respectively). Douglas (1980) reported 0 of 12 pregnancies for embryos recovered and transferred in TCM-199 compared with 6 of 10 pregnancies for embryos collected and transferred in Dulbecco's phosphate buffered saline (DPBS). In contrast, others have reported that TCM 199 maintained viability of embryos (Allen and Rowson, 1975). Equine embryos held at room temperature in DPBS for 6 to 24 h prior to transfer resulted in 3 of 7 pregnancies at 24 d and 0 of 7 pregnancies at 100 d compared with 20 of 28 and 14 of 28, respectively, for control embryos that were held for less than 3 h in DPBS at room temperature (Douglas, 1982). Studies in our laboratory also demonstrated that DPBS was incapable of maintaining embryo viability for 24 h (Imel, 1981), whereas Ham's F10 was a better medium for equine embryos. Slade et al. (1984) bisected equine embryos and stored one-half of each of five pairs in either Ham's F10 + 10% fetal calf serum (FCS) in 5% CO2 in air at 37 C or DPBS + 10% FCS in air at 37 C. This preliminary study indicated that Ham's F10 was superior (P<.10) to DPBS in promoting growth and development while maintaining the quality of the equine demembranates. However, use of a nonbicarbonate buffered medium would be more practical under field conditions. Therefore, studies were conducted to evaluate various culture systems for equine embryos.

Materials and Methods

General. Mares used in this study were nonlactating light-horse type mares. Each mare was fed 2 to 4 kg of a grain mixture and alfalfa hay (2.5% of body weight). Mares were maintained outside in pasture in groups of 50 to 80. Estrus detection was performed daily with a teaser stallion. All mares in estrus were examined per rectum for follicular development and detection of ovulation once (Exp. 2 and 3) or twice (Exp. 1) daily until ovulation. Upon detection of a follicle >35 mm, mares designated as embryo donors were inseminated every-other-day with 250 × 10^6 progressively motile spermatozoa from one of two stallions of known fertility. Upon detection of ovulation (d 0) donor mares were scheduled for embryo recovery on d 6.5 (Exp. 1) or d 6 (Exp. 2 and 3). Embryo recovery was performed nonsurgically as described by Squires et al. (1985a). Upon location, the embryo was transferred to a "holding" medium of DPBS + 10% FCS at room temperature for <1 h until placed into the assigned culture medium.

Exp. 1. The objectives of this study were: 1) to develop an in vitro culture system that would maintain viability of equine embryos for 24 h and 2) specifically to compare viability of equine embryos stored in DPBS, Ham's F10 and Minimal Essential Medium (MEM) for 24 h.

Embryos >200 µm were randomly assigned to one of three treatments (n=10/group): 1) Ham's F10 + 10% FCS under 5% CO2, 5% O2, 90% N2 at 24 C; 2) MEM with Hank's balanced salts + 10% FCS in air at 24 C (MEM) and 3) MEM at 5 C.

Embryos less than 200 µm were microsurgically bisected (Williams et al., 1984). One-half of the embryo remained in the original zona pellucida, while the other half was transferred into a surrogate bovine zona pellucida. If a capsule was present, the capsule was replaced inside the original zona pellucida. One-half of each embryo was assigned to the control medium, Ham's F10 at 24 C (n=10/group), whereas the other half was randomly assigned to one of two treatments (n=10): 1) DPBS in air at 24 C or 2) MEM in air at 24 C.

Media were prepared by addition of deionized water (18.3 megohms) to dry chemicals. Each solution was sterilized by filtration through a 0.22-µm millipore filter after mixing and stored without FCS supplement for up to 2 mo at 4 C. A 10% v/v solution of FCS: medium was prepared for each embryo. Media containing FCS were millipore-filtered through a 0.22-µm syringe filter and stored up to 1 wk.

Embryos were drawn into sterile, fire-polished pipettes and "washed" by movement through three drops of the assigned culture medium containing 10% heat-treated FCS. Embryos were cultured in 15- x 60-mm culture dishes. A microdrop (approximately 100 µl) of the assigned medium was pipetted beneath a film of paraffin oil and the embryo was pipetted into the microdrop. One drop was placed in each dish and only one embryo was placed in a drop. Dishes containing embryos assigned to DPBS or MEM at 24 C were placed in a 24 C incubator in an air-conditioned room. Embryos assigned to the MEM medium at 5 C were placed in a
“walk-in” cooler maintained at 5 C. Dishes that were to contain embryos in Ham’s F10 were prepared in advance and allowed to equilibrate for several hours at 37 C under an atmosphere of 5% CO2 in air. Paraffin oil to cover drops of the Ham’s medium was also previously gassed with 5% CO2. The dish containing an embryo was placed on top of an inverted plastic cup that had perforations on the lateral surface. The cup was placed in a GasPak jar. A damp paper towel was placed beneath the inverted cup to maintain humidity at 100%; the lid was placed on top of the jar and a gas mixture of 5% CO2, 5% O2 and 90% N2 was passed into the canister for 1 min. Following gassing, the canister was placed in a 24 C incubator in an air-conditioned room. The canister was re-gassed after each evaluation.

**Embryo Evaluation.** Embryos were evaluated upon initial location in the search dish (time 0), again after bisection for those less than 200 μm, and at 12 and 24 h after placement into the culture medium. At each time, embryos were measured with a Filar eyepiece micrometer and evaluated for stage of development and quality. Embryos were assigned a developmental score of 1 to 4: 1-tight morula, 2-early blastocyst, 3-blastocyst and 4-expanding blastocyst and a quality score of 1 to 5 (1 = excellent, 5 = degenerate). The quality scoring system was an adaptation of that used to evaluate bovine embryos (Elsden et al., 1978; Kuzan, 1983) and was based on evaluation of the following morphologic characteristics: 1) compactness of blastomeres, 2) extrusion of blastomeres, 3) color of embryo, 4) embryo shape, 5) damage to zona pellucida/capsule and 6) proportion of the perivitelline space occupied by the cell mass.

Embryos were also evaluated on their ability to develop/grow in culture. Embryos were judged to have developed in culture if they advanced from one developmental stage to the next, e.g., if an early blastocyst progressed to a blastocyst. Embryos classified as non-developing did not grow into a more advanced stage and/or showed various degrees of degeneration.

**Staining of Embryos.** After 24 h in culture, all embryos were fixed and stained. The embryo in a drop of medium was pipetted onto a slide between two strips of petroleum jelly. A coverslip was placed over the embryo and petroleum jelly strips and then pressed down upon the embryo until it was held firmly in place. Fixative (3:1 95% ethanol: acetic acid) was drawn under the coverslip by using strips of filter paper. Slides were then placed flat in a petri dish, covered with fixative, and left for 24 to 48 h. Aceto-orcein was prepared by addition of 1 g orcein to 100 ml of 45% glacial acetic acid and warmed until the orcein was dissolved. Embryos were stained with the aceto-orcein for 10 to 20 min, after which excess stain was rinsed away with 45% glacial acetic acid, and the edges of the coverslip were sealed with fingernail polish to prevent the preparation from drying out. Within 24 h of staining, dark-staining cells (dead cells) were counted at 450× magnification using a brightfield microscope.

**Exp. 2.** The objective of this experiment was to evaluate viability of embryos stored in Ham’s F10 at 5, 24 or 37 C. The procedures for handling donor mares and embryo recovery were as described previously.

Recovered embryos were drawn into sterile, fire-polished pipettes and washed through three drops of millipore-filtered DPBS with 10% FCS and held at room temperature (< 1 h) in a 10-× 35-mm dish until placed into the experimental culture media. A table of random numbers was used to assign embryos to one of three treatments (n=10/treatment). Embryos were cultured in Ham’s F10 plus 10% FCS in 5% CO2, 5% O2 and 90% N2 and held at one of three temperatures (5, 24 or 37 C) for the initial 12 h of storage. During the final 12 h all embryos were cultured at 37 C.

Preparation of Ham’s F10 was as described for Exp. 1. Embryos were pipetted from the DPBS “holding medium” and passed through three drops of millipore-filtered DPBS with 10% FCS and held at room temperature (< 1 h) in a 10-× 35-mm dish until placed into the experimental culture media. A table of random numbers was used to assign embryos to one of three treatments (n=10/treatment). Embryos were cultured in Ham’s F10 plus 10% FCS in 5% CO2, 5% O2 and 90% N2 and held at one of three temperatures (5, 24 or 37 C) for the initial 12 h of storage. During the final 12 h all embryos were cultured at 37 C.

Embryos were evaluated, as described for Exp. 1, at 0, 12 and 24 h after culture. Embryo development was determined and scored on the scale of 1 to 4, whereas quality of the embryos was assessed and scored on the scale of 1 to 5. Diameter was determined by measurements with a Filar eyepiece micrometer and recorded in micrometers. Development in culture was determined by increase in size and/or advancement in developmental stage.
Exp. 3. The objectives were: 1) to determine if embryos stored in Ham's F10 at 24 C for 12 h and transferred nonsurgically to recipient mares would result in equal pregnancy rates compared with embryos transferred in <1 h after collection and 2) to determine pregnancy rates in progesterin-treated recipients compared with naturally synchronized recipients.

A table of random numbers was used to assign 60 embryos to one of four treatments: 1) embryo transferred <1 h into a naturally synchronized recipient that had ovulated 1 d prior to or within 3 d after the donor; 2) embryo transferred <1 h into a progesterin-treated recipient; 3) embryo transferred, after 12 h storage at Ham's F10, into a naturally synchronized recipient that ovulated between 1 d before to 3 d after the donor and 4) embryo transferred after 12 h storage in Ham's F10, into a progesterin-treated recipient. If the donor did not provide an embryo or an appropriate recipient was not available, that particular cell was filled later.

Recipients used for embryos assigned to treatments 2 and 4 were prepared as follows: when a donor ovulated, a recipient mare between d 3 and 12 of diestrus was identified and administered .044 mg of altrenogest per kg body weight daily, beginning the day after the donor mare's ovulation. Treatment was continued until d 30 if pregnancy was confirmed, but was discontinued at d 20 if the embryo failed to establish a pregnancy. Culture medium was prepared in the same manner as described for the previous two experiments. Each embryo was washed through three drops of Ham's F10, placed into a 10- x 35-mm culture dish, evaluated and given a quality and developmental stage score, and its diameter was measured. The embryo was then loaded into a .5-ml straw. Medium was aspirated into the straw, then a column of air, then fluid with the embryos followed by a small column of air. A film of paraffin oil was pipetted above the final column of medium. The straw was placed in a vertical position for 2 min to allow the embryo to settle toward the cotton-plugged end. The distal end (above the paraffin oil) was then heat-sealed. The straw was placed into a 16- x 125-mm test tube containing 4 ml of Ham's F10 + 10% FCS that had previously been stored at 37 C in 5% CO2. The test tube was then placed, at a 45° angle, in a GasPak jar with a moist paper towel set in the base. The canister was then gassed with a 5% CO2, 5% O2 and 90% N2 mixture and the lid was secured. The canister was placed in an incubator that had equilibrated to 24 C in an air-conditioned room. Embryos were evaluated for size, developmental stage and quality at time 0 and after 12 h of storage.

Embryos were transferred nonsurgically into the uterine body using a telescopic embryo transfer instrument, as described previously (Squires et al. 1985b). Pregnancy detection was performed by ultrasonic scanning of the uterus per rectum. Recipient mares were scheduled for their initial scan when the embryo attained 12 d of age. Mares were also scanned on d 14, 16 and 20 regardless of the results of previous examinations. Mares contained to be scanned on d 25 and 30 if a vesicle was detected. When a vesicle was observed, its largest diameter was measured and recorded. At 33 d of gestation, the recipient was administered 7.5 mg prostaglandin F2α intramuscularly to induce abortion. Recipients that aborted and experienced a subsequent normal estrous cycle were reused, providing the uterus was normal in size and devoid of ultrasonically visible fluid.

Statistical Analyses. For quantitative data obtained in Exp. 1 and 2, analyses of variance for repeated measurements were used. When the F-statistic was significant, treatment means were compared by the Least Significant Difference procedure, using the appropriate error for a split-plot design. Numbers of embryos in each group advancing in development during the culture period were compared by chi-square analysis (Steel and Torrie, 1980). In Exp. 3, differences between developmental stage, quality and diameter of embryos prior to treatments were analyzed by chi-square analysis (Steel and Torrie, 1980). These same characteristics for embryos in treatments 3 and 4 were analyzed in a subsequent analysis to determine changes from 0 to 12 h. Number of mares becoming pregnant in each of the four treatments was evaluated by chi-square analysis to test for homogeneity of proportions. Fisher's exact test for 2 x 2 tables was used to test for homogeneity of pregnancy proportions when the sample size was small (McGuire et al. 1967). A two-tailed Fisher's exact test was used to determine
the relationship between developmental stage, quality, diameter and transfer score and the number of pregnancies obtained in treatments 1 and 3.

Results and Discussion

Exp. 1. At time 0, whole embryos stored in MEM at 5 C were at a more (P<.05) advanced stage of development (3.2) than those stored in MEM at 24 C (2.4) or in Ham's F10 at 24 C (2.0). This was apparently due to chance, since embryos were randomly assigned to treatments. Embryos stored in MEM at either 5 or 24 C did not (P>.05) change in stage of development over the 24-h period of culture. In contrast, embryos stored in Ham's at 24 C were at a more (P<.05) advanced stage of development after 24 h than at time 0 (2.0 vs 2.6).

The number of whole embryos that advanced in stage of development was similar (P>.05) among the three treatments from 0 to 12 and 12 to 24 h (table 1). However, fewer embryos developed between 0 and 24 h in MEM at 5 C than in MEM at 24 C and Ham's F10 at 24 C (P<.05).

Embryo quality was similar (P>.05) among treatments at times 0 and 12 h (table 2). However, by 24 h, embryo quality was better (P<.05) for embryos stored in Ham's F10 compared with those stored in MEM at 5 C. Embryo quality deteriorated over time for those stored in MEM at 5 and 24 C but not for embryos cultured in Ham's F10 at 24 C. Deterioration in quality of embryos cultured in MEM at 24 C occurred during the initial 12 h, with a quality score of "good" being maintained through the final 12 h of culture.

Embryos stored at 5 C in MEM appeared slightly shrunken away from the zona pellucida by 12 h in culture; by 24 h the layer of trophoblast cells appeared rough, suggesting damage to the tight junctions between these cells. These cells were also slightly darker than when initially placed into culture, suggesting cell death. Neither culture media nor incubation time affected embryo diameter or percent change in size.

Stage of development of bisected embryos was similar (P>.05) at each time period for those stored in either Ham's F10 or MEM (table 3). By 24 h, halved embryos in both of these treatments had progressed in development (P<.05). After 12 h of culture demi-embryos stored in Ham's F10 or DPBS advanced in development. At both 12 and 24 h, embryos cultured in Ham's F10 were at a more (P<.05) advanced stage of development than those stored in DPBS (table 3).

Presented in table 4 are the quality scores of bisected embryos throughout the 24-h culture period. Demi-embryos stored in MEM had a poorer (P<.05) quality score at both the 12- and 24-h period than those in Ham's F10. Embryos stored in Ham's F10 maintained slightly less than "excellent" quality scores throughout the 24-h period. Bisected embryos stored in the DPBS treatment deteriorated throughout the 24-h period (table 4). Overall, quality scores were superior (P<.05) for halved embryos stored in Ham's F10 (1.5) compared with DPBS (1.8).

Diameters of bisected embryos increased (P<.05) across all treatments during the initial 12 h in culture. However, there were no differences (P>.05) in embryo diameter between any of the treatments. Initial rapid growth following bisection was expected, because demi-embryos usually regain their previous developmental stage.

The equine blastocyst contains hundreds of cells, and by the end of the culture period,

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Treatment (n=10/treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12</td>
<td>MEM 5 C</td>
</tr>
<tr>
<td>4/10</td>
<td>2/10</td>
</tr>
<tr>
<td>12-24</td>
<td>0/10</td>
</tr>
<tr>
<td>0-24</td>
<td>1/10</td>
</tr>
</tbody>
</table>

*aNumber of embryos that advanced in stage of development at each time period.

bχ² = 8.3 (P<.05), 2 df.
TABLE 2. EFFECT OF CULTURE MEDIA ON QUALITY OF EQUINE EMBRYOS

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Treatment (n=10/treatment)bc</th>
<th>MEM 5 C</th>
<th>MEM 24 C</th>
<th>Ham's F10 24 C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2d</td>
<td>1.2d</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.6e</td>
<td>1.8e</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2.2fg</td>
<td>1.8gh</td>
<td>1.4h</td>
<td></td>
</tr>
</tbody>
</table>

a = excellent, 2 = good, 3 = fair, 4 = poor, 5 = degenerate.
b = Error term for media treatment was .78 with 27 df.
c = Error term for time or treatment X time was .12 with 54 df.
d, e, f = Means within columns with different superscripts differ (P<.05).
g, h = Means within rows without a common superscript differ (P<.05).

most of the embryos were at the blastocyst stage. The large number and small size of individual blastomeres composing the blastocyst contributed to a futile attempt at enumerating pyknotic nuclei. Mounting of the embryo on a slide, for purposes of staining, distorted many of the cells, causing difficulty in distinguishing individual cells. Furthermore, differentiating a stained cell from layers of cells superimposed on each other was extremely difficult. Thus, post-culture evaluation of the equine embryo based on staining properties of the blastomeres was omitted.

Exp. 2. Embryos cultured at 5 or 24 C for the initial 12-h period did not (P>.05) advance in development. However, once embryos in these two treatments were cultured at 37 C, they advanced in development. Embryos cultured at 37 C throughout the 24-h period had advanced (P<.05) in development at both the 12 and 24 h evaluations. Averaged across all treatments, embryos advanced in developmental at each period (table 5).

Although embryos cultured at 37 C had developed after 12 h, developmental stage score for these embryos did not differ (P>.05) from embryos at 5 or 24 C. However, embryos assigned to the 37 C treatment were initially (time 0) at a less-developed stage (2.2, P<.05) than those stored at either 5 C (2.8) or 24 C (2.6). This may explain why no differences were detected among treatments even though embryos stored at 37 C advanced one unit in development (2.2 to 3.2) compared with .5 units for the 5 C treatment and .4 units for the 24 C treatment. Although embryos developed

TABLE 3. EFFECT OF CULTURE MEDIA ON DEVELOPMENT SCORE OF EQUINE BISECTED EMBRYOS

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Treatment (n=10/treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ham's F10 24 C</td>
</tr>
<tr>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>12</td>
<td>1.8</td>
</tr>
<tr>
<td>24</td>
<td>2.0</td>
</tr>
</tbody>
</table>

a = tight morula, 2 = early blastocyst, 3 = blastocyst, 4 = expanding blastocyst.
b = Error term for media treatment was .25 with 9 df. Error term for time or treatment X time was .14 with 18 df.
c = Error term for media treatment was .10 with 9 df. Error term for time or treatment X time was .15 with 18 df.
d, e, f = Means within columns without a common superscript differ (P<.05).
f, g = Means within rows with different superscripts differ (P<.05).
**TABLE 4. EFFECT OF CULTURE MEDIA ON QUALITY SCORE OF EQUINE BISECTED EMBRYOS**

<table>
<thead>
<tr>
<th>Treatment (n=10/treatment)</th>
<th>Ham's F10</th>
<th>MEM</th>
<th>Ham's F10</th>
<th>DPBS</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, h</td>
<td>24 C</td>
<td>24 C</td>
<td>24 C</td>
<td>24 C</td>
<td>Mean</td>
</tr>
<tr>
<td>0</td>
<td>1.4</td>
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</tr>
<tr>
<td>12</td>
<td>1.4</td>
<td>1.6</td>
<td>1.6</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>24</td>
<td>1.9</td>
<td>1.7</td>
<td>1.7</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Mean</td>
<td>1.4</td>
<td>1.7</td>
<td>1.7</td>
<td>1.8</td>
<td>1.7</td>
</tr>
</tbody>
</table>

a = excellent, 2 = good, 3 = fair, 4 = poor, 5 = degenerate.

b = Error term for media treatment was .35 with 9 df. Error term for time or treatment X time was .15 with 18 df.

c = Error term for media treatment was .20 with 9 df. Error term for time or treatment X time was .11 with 18 df.

d, e = Means within columns with different superscripts differ (P<.05).

f, g = Means within columns with different superscripts differ (P<.10).

h = Means within rows with different superscripts differ (P<.05).

at a minimal rate when held at 5 or 24 C, these embryos were capable of normal development upon being cultured at 37 C. Apparently, a temperature of 24 C is just as inhibitory to development of an equine embryo as culture at 5 C.

Quality of embryos in all treatments did not deteriorate (P>.05) during the 24-h culture period. Embryos held at 5 C did not change in quality score by 12 h. Although embryos stored at 24 C tended (P<.10) to deteriorate, subsequent culture of these same embryos at 37 C improved quality score similar to that obtained prior to storage. Embryos cultured for the entire 24-h period at 37 C maintained a quality score of "excellent" (1.2).

Growth of embryos during the initial 12-h period was minimal for those stored at 5 or 24 C (1.5 and 9.3% increase in diameter, respectively). However, one these embryos were cultured in Ham's F10 at 37 C, a dramatic increase in size was noted (37 and 30%, respectively). Embryos cultured in 37 C for the entire period increased in size during both 12-h culture periods (0 to 12 h, 33%; 12 to 24 h, 37%). Although not significantly different, embryo size was less at time 0 for those in the 37 C treatment (187 μm) compared with the mean size.

**TABLE 5. EFFECT OF STORAGE TEMPERATURE ON DEVELOPMENTAL STAGE SCORE OF EQUINE EMBRYOS**

<table>
<thead>
<tr>
<th>Treatment (n=10/treatment)</th>
<th>Ham's F10</th>
<th>Ham's F10</th>
<th>Ham's F10</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, h</td>
<td>5 C</td>
<td>24 C</td>
<td>37 C</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.8</td>
<td>2.6</td>
<td>2.2</td>
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<tr>
<td>12</td>
<td>3.0</td>
<td>2.8</td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>24</td>
<td>3.3</td>
<td>3.0</td>
<td>3.2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

a = tight morula, 2 = early blastocyst, 3 = blastocyst, 4 = expanding blastocyst.

b = Error term for media treatment was .35 with 27 df.

c = Error term for time or treatment X time was .10 with 54 df.

d, e, f = Means within columns without a common superscript differ (P<.05).

g, h = Means within rows with different superscripts differ (P<.05).
size for embryos assigned to the 5 C (254 μm) or 24 C (216 μm) treatments.

Once embryos were stored at 37 C, the percent increase in size was similar (P>.05), regardless of the prior incubation temperature. Overall, embryos stored at 37 C for the final 12-h period increased in diameter by 35%. Thus, it appeared that embryos could be held for a period of at least 12 h at a reduced temperature without altering their capability to develop when subsequently cultured at a higher temperature. Refrigeration temperature (5 C), for a 12-h period, arrested development but prevented deterioration of embryo quality. Thus, this treatment might be a suitable means for transporting equine embryos. Although embryos held at room temperature (24 C) showed some increase in developmental stage and diameter, they tended to deteriorate in quality. However, this deterioration in quality did not prevent embryo development upon culture at 37 C. Thus, storage of embryos lower than body temperature could be used “slow down” the growth of equine embryos. Equine embryos can be stored for at least 12 h at 5, 24 or 37 C and still remain viable.

Exp. 3. Embryos assigned to the four treatments were similar (P>.05) in quality, developmental stage and size at time 0. Data for embryos in treatments 3 and 4 were combined and tested for the main effect of time (0 vs 12 h). Embryos advanced (P<.05) in development and size, while quality deteriorated slightly (P<.05) during the 12-h culture period in Ham's F10 at 24 C. However, the majority of embryos were graded slightly better than “good” (1.7) by 12 h, although four embryos deteriorated to a grade of fair to poor quality. Most embryos that were pre-blastocysts attained the blastocyst stage by 12 h. Mean diameter was 247 μm at the beginning of culture and 281 μm 12 h after storage.

Fifteen of 32 embryos transferred to naturally synchronized recipients established a pregnancy, as detected by ultrasound on d 12 (6 d after transfer of the embryo). There was no difference (P>.05) in pregnancy rates for those embryos that had been stored for 12 h in Ham's F10 at 24 C (8 of 16) compared with those transferred within 1 h of collection (7 of 16).

Embryos transferred into progesterin-treated recipients resulted in fewer (P<.05) pregnancies (2 of 17) compared with those transferred into synchronized recipients. Because of the poor success with the use of progesterin-treated recipients, these treatments were discontinued after only eight and nine transfers, and these data were not included in subsequent analyses. Overall, there was no difference (P>.05) in pregnancy rates for embryos stored for 12 h (9 of 25) compared with those transferred immediately (8 of 24).

One embryonic death was detected in each of the four treatments, so that by 30 d, none of the progesterin-treated mares remained pregnant, and 13 of 32 (40.6%) naturally synchronized recipients remained pregnant. This nonsurgical pregnancy rate of 40.6% is lower than that reported previously in our laboratory (Squires et al., 1985b), even though the same technician and methods were used in both studies. A more stringent selection of recipients in the previous study may have contributed to this apparent difference in pregnancy rate.

The reason for failure of progesterin-treated recipients to establish a pregnancy is unclear. After nine unsuccessful transfers, the dose of altrenogest was increased to .088 mg/kg body weight. Eight recipients were treated with the high dose of altrenogest. Two recipients treated with the high dose became pregnant, but both embryos were lost by d 20. These embryos were not detected by ultrasonic scanning until d 16. Both vesicles measured half the size of those of a normal pregnancy of the same age. Recent studies in our laboratory (A. O. McKinnon, unpublished) demonstrated that transfer of embryos into ovariectomized mares administered .044 mg altrenogest/kg body weight resulted in 14 of 20 pregnancies. Others have also reported on use of ovariectomized-progesterone treated mares as embryo transfer recipients (Hinrichs et al., 1985, 1986). In both studies involving ovariectomized mares, altrenogest or progesterone was administered for only 5 to 7 d prior to transfer, whereas in the present study some recipients had been exposed to endogenous progesterone for 3 to 12 d prior to receiving exogenous progesterin for 5 to 6 d. Apparently this extended exposure of the uterus to progesterins resulted in an unfavorable uterine environment. Recent studies in both intact (Pool et al., 1986; Parry-Weeks and Holtan, 1986) and ovariectomized mares (Hinrichs et al., 1986) provided further evidence that progesterin treatment for longer than approximately 9 d prior to embryo transfer resulted in poor pregnancy rates. Perhaps the exposure to progesterin advanced the endometrial development of the uterus such that there was an asynchrony between the embryo and endometrial development.
After five transfers, a decision was made to score each transfer based on a code of 1 to 4 (1 = excellent, 4 = poor). An excellent score was assigned to those transfers performed quickly with no difficulty. A score of 4 was assigned to a transfer that required considerable time, excessive straining of the mare and difficulty in extending the straw. For analysis, the transfer score was categorized into scores of 2 or less and greater than 2. There appeared to be a relationship between transfer score and pregnancy rate. Scores of <2 (n = 24) resulted in 13 pregnancies, while none of the three embryos receiving a score of >2 resulted in a pregnancy. Although a pregnancy resulted from 54% of the transfers rated <2, the number of transfers performed receiving a score >2 was too small to allow a meaningful conclusion.

The developmental stage of the embryo at the time of transfer influenced pregnancy for embryos stored for 12 h (treatment 3) but not for those embryos transferred immediately (treatment 1). Cultured embryos that had not advanced to a developmental score of 2.5 did not establish a pregnancy (0 of 7) compared with eight of nine pregnancies for embryos with a score of >2.5 prior to transfer. A score of 2.5 corresponded to an embryo slightly beyond the early blastocyst stage. The mean developmental score for stored embryos resulting in a pregnancy was 3.2 compared with 1.5 for embryos that failed to establish a pregnancy.

Although studies in cattle (Elsden et al., 1978) have demonstrated a relationship between embryo quality and pregnancy rates after transfer, apparently no similar studies are available for equine embryos. Data for embryos in the present study were categorized retrospectively into either a quality score <1.5 or >2.0. Of the 26 embryos assigned a quality score of <1.5 prior to transfer, 13 (50%) resulted in a pregnancy. In contrast, of the six embryos transferred with a quality score >2 only one pregnancy was established. Although there was a tendency for higher quality embryos to result in more pregnancies, there were insufficient numbers of lower quality embryos for this difference to be significant.

Embryo size did not affect the overall number of pregnancies obtained. Six of 17 embryos <175 μm resulted in pregnancies vs 9 of 15 pregnancies for embryos >175 μm. In treatment 3, greater numbers of pregnancies were obtained from embryos >175 μm than <175 μm (seven of nine vs one of seven). Thus, less developed, and therefore smaller embryos, appear to be more susceptible to the damaging effect of reduced temperature storage.

In summary, results of these studies have shown that embryo viability can be maintained for 12 h by storage of equine embryos in Ham’s F10 at 24°C.

Literature Cited


