EMBRYO TRANSFER AS A MEANS OF CONTROLLING THE TRANSMISSION OF VIRAL INFECTIONS.
II. THE IN VITRO EXPOSURE OF PREIMPLANTATION BOVINE EMBRYOS TO INFECTION BOVINE RHINOTRACHEITIS VIRUS

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ABSTRACT

Bovine embryos, at the 16-cell to the blastocyst stage of development, were exposed to infectious bovine rhinotracheitis virus (IBRV) for either one or 24 hours. These embryos were then washed and incubated for 24 or 48 hours before being assayed for IBRV. Under these conditions, infectious virus at the level of $0-10^{2.2} \text{TCID}_{50}/\text{ml}$ was isolated from 57-64% of the embryos exposed to IBRV. Trypsin and IBRV-antiserum were found to be capable of removing and/or inactivating the IBRV from exposed embryos. Both the low level of the virus isolated from these embryos and the susceptibility of this virus to trypsin and antiserum suggests that IBRV attaches to the zona pellucida of embryos and cannot penetrate this structure to gain access to the embryonic cells. IBRV was found to have no effect on embryonic development in vitro. In addition, thirty-one eggs/embryos isolated from donors that were seropositive to IBRV were found to be uninfected with this virus.

INTRODUCTION

The requirements for using embryo transfer for disease control have been discussed previously (1). Briefly, embryo transfer could be used to prevent the transmission of a viral infection if the virus were not present in the gametes and if preimplantation embryos were not susceptible to infection (or did not passively carry the virus) prior to their being collected and transferred to recipient animals. Since few viruses have been isolated from either ova or spermatozoa (1), it is likely that the majority of prenatal infections result from the presence of the virus in the embryonic environment rather than in the gametes. It is therefore important to determine the susceptibility of preimplantation embryos to viral infections.

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A previous paper (2) describes the preliminary in vitro experiments that were carried out to assess embryo transfer as a means of controlling bluetongue, akabane, and bovine viral diarrhea viruses. This study deals with the in vitro susceptibility of bovine embryos to infectious bovine rhinotracheitis virus (IBRV).

IBRV, a herpes virus responsible for respiratory disease, venereal infection and abortion in cattle, is readily transmissible and is of major economic importance to Northern Hemisphere countries and Australia. The virus can persist for a considerable period of time in recovered animals and, under certain circumstances, can cause recurrent disease (3,4). Aborted bovine fetuses have been shown to be infected with IBRV (5-7), but the earliest stage at which the embryo/fetus can become infected 'in utero' has not been determined. If embryo transfer is to be used to control or eliminate IBRV, it is essential to determine whether early bovine embryos can be infected with this virus. If they cannot and IBR is not carried in the gametes, then control of IBRV using embryo transfer would be possible.

**MATERIALS AND METHODS**

I. Embryos from IBRV seronegative donors

Embryos were collected non-surgically on days 5,6 or 7 from superovulated IBRV-seronegative donors as previously described (8). After collection, the embryos were assessed microscopically in terms of their stage of development and condition prior to being treated.

**Virus**

Infectious bovine rhinotracheitis virus (IBRV, "Colorado strain") was passaged in primary fetal bovine kidney (FBK) cells. Samples were assayed for infectivity in passaged FBK cells by cytopathic effect (CPE) in microtiter plates and by plaque assay under an overlay containing MEM, 0.8% agar and 10% fetal bovine serum (FBS) (9).

**Treatment of embryos**

Viral exposure. Embryos from each donor were transferred into 100 μl drops of IBRV-infected or control media (lysates of uninfected cell cultures) and then incubated in a water saturated atmosphere of 5% CO₂ in air for 1 or 24 hours. After incubation, the embryos (in groups of 8 or less) were washed by passing them through ten 100 μl drops (in another 100 x 15 mm petri dish) of Brinster's BMOC-3 medium(a). A fresh micropipette was used to transfer the embryos to each successive drop. After being exposed to the virus and washed, some embryos were

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(a)GIBCO, 3175 Staley Road, Grand Island, New York 14072
immediately assayed to detect any attached virus (zero hour controls). The remaining virus-exposed embryos and the control embryos were cultured individually in microtitre plates for 24 or 48 hours in 100 μl volumes of culture medium in a water saturated atmosphere of 5% CO₂ in air prior to being assayed. The culture medium (CM) was Ham's F10 supplemented with 20% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin.

Of a total of 83 bovine embryos exposed to IBRV and then washed: 15 embryos were assayed immediately (zero hour controls), 54 were assayed after 24 hours culture, and 14 were assayed after 48 hours culture (Table 1).

Antiserum treatment. An additional group of 71 embryos was exposed to $10^6$–$10^8$ TCID₅₀/ml of IBRV for 24 hours and then washed. Thirty-five of these embryos were then incubated for either 30 minutes or 1 hour in Ham's F10 medium containing 10% guinea pig complement and IBRV-antiserum diluted 1:5. The antiserum used in these experiments neutralized $10^5$ TCID₅₀/ml of IBRV when diluted 1:5. These 35 embryos were then washed through 5 drops of CM and cultured. The remaining 36 embryos exposed to IBRV, but not to antiserum (antiserum control embryos), were also transferred through 5 drops of CM prior to culturing. After 24 hours, the effect of antiserum on embryonic development was assessed microscopically by comparing the embryos treated with antiserum with those untreated. All 71 embryos were then assayed for IBRV (Table 3).

Trypsin treatment. A separate group of 59 embryos was exposed to $10^6$–$10^8$ TCID₅₀/ml of IBRV for 24 hours and then washed. Thirty-two of these embryos were then treated with 0.25% trypsin for 1-2 minutes at 37°C. They were then transferred through 5 drops of CM to remove the trypsin and cultured. The remaining 27 embryos exposed to IBRV, but not to trypsin (trypsin-control embryos), were also transferred through 5 drops of CM prior to culturing. After 24 hours, the effect of trypsin on embryonic development was assessed by comparing the 32 embryos treated with trypsin with the 27 that were not treated. All of these embryos were then assayed for IBRV (Table 3).

Effect of viral exposure on embryonic development

The effect of viral exposure on embryonic development was assessed after culturing for 24 to 48 hours by comparing

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(a) All serum used in this study was seronegative for IBRV

(b) GIBCO, 3175 Staley Road, Grand Island, New York 14072
56 embryos exposed to the virus with 40 embryos that were not exposed. These 56 embryos were from the same donors and were judged, prior to viral exposure, to be at the same stages of development and condition as the 40 embryos used as controls.

**Preparation of samples for viral assays**

Samples to be assayed were transferred to 12 x 75 mm capped plastic tubes containing 1 ml of 4°C PBS. The embryos, the virus to which the embryos were exposed, the drops of medium in which the embryos were washed, and the medium in which the embryos were cultured were all assayed for the presence of the virus. Embryos were sonically disrupted on ice with three 5 sec pulses at 10 kc with an ultrasonic oscillator. The majority of samples were assayed immediately to prevent any loss of virus during freezing.

**II. Embryos from IBRV seropositive donors**

Thirteen embryos and eighteen unfertilized eggs were available from seven donors seropositive for IBRV. These embryos/eggs were collected on days 5, 6 or 7 and, without further treatment, they were assayed (as above) for infection with IBRV.

**RESULTS**

Infectious virus was isolated from 57% (8/14) and 64% (35/54) of embryos exposed to IBRV for 1 and 24 hours, respectively (Table 1). There was no relationship between embryos with infectious virus and the developmental stage of the embryo, which ranged from the 16-cell to

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Exposure of preimplantation bovine embryos to 10⁶-⁸TCID₅₀/ml of IBRV</th>
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<tr>
<td></td>
<td>No. Embryos</td>
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<tr>
<td>Zero hr control (a)</td>
<td>15</td>
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<tr>
<td>1 hr virus exposure (b)</td>
<td>14</td>
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<tr>
<td>24 hr virus exposure (b)</td>
<td>54</td>
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(a) Zero hr control embryos were exposed to the virus for either 1 or 24 hrs, washed and then assayed directly.

(b) Embryos exposed to the virus for 1 hour were washed and cultured for 48 hours before being assayed. Those exposed for 24 hours were washed and cultured for 24 hours prior to being assayed.
the blastocyst stage. Assays of the washes of the embryos after viral exposure showed that the first 6-7 washes contained decreasing amounts of infectious virus and that the last 3-4 were always negative. Re-assay of the virus after sample processing showed that there was no loss of infectivity of IBRV.

After 24 and 48 hours of culture, all embryos were still within the zona pellucida. No difference was found in the in vitro embryonic development of embryos exposed to IBRV compared with unexposed controls (Table 2).

Attempts to "disinfect" the embryos with additional washings proved unsuccessful. Increasing the number of washings from ten to twenty failed to reduce either the number of embryos carrying infectious virus or the level of infection in/on the embryos. However, both IBRV-antiserum and trypsin were found to be capable of removing infectious virus from IBRV-exposed embryos (Table 3).

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<thead>
<tr>
<th>Table 2</th>
<th>Effect of IBRV on embryonic development on bovine embryos in vitro</th>
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<tr>
<td>Control Embryos</td>
<td>Embryos Exposed to Virus</td>
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<td>No. cultured (% with continued in vitro development)</td>
<td>No. cultured (% with in vitro development after viral exposure)</td>
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<tr>
<td>16-cell</td>
<td>morula</td>
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<td>16 (75%)</td>
<td>13 (84%)</td>
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<tr>
<th>Table 3</th>
<th>Antiserum and trypsin treatment of embryos exposed to 10^6-8 TCID50/ml IBRV</th>
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<tr>
<td>No antiserum</td>
<td>3</td>
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<tr>
<td>Antiserum for 1/2 hr</td>
<td>3</td>
</tr>
<tr>
<td>No antiserum</td>
<td>4</td>
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<tr>
<td>Antiserum for 1 hr</td>
<td>4</td>
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<tr>
<td>No trypsin</td>
<td>4</td>
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<tr>
<td>Trypsin for 1-2 min</td>
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</table>
The eighteen unfertilized eggs and thirteen embryos from donors that were seropositive for IBRV were found to be uninfected with IBRV.

**DISCUSSION**

Although IBRV had no effect on embryonic development in vitro (Table 2), over half of the embryos exposed to IBRV yielded infectious virus (Table 1). Since the level of infection from embryos cultured for 24 or 48 hours was the same as embryos assayed immediately after washing (zero hour controls), embryonic infection with viral replication had probably not occurred. This lack of viral replication might be explained by the failure of IBRV to replicate in the relatively undifferentiated cells of the embryo. If this is the case, and IBRV is capable of crossing the zona pellucida of early embryos, then it would not be possible to prevent the transmission of IBRV using embryo transfer. The virus would be transferred along with the embryo and it is likely that with increasing embryonic differentiation, IBRV would prove capable of replicating in the embryo.

However, it is also possible that IBRV cannot penetrate the zona pellucida of early embryos and that it merely attaches to this structure. In terms of disease transmission, virus passively carried by the embryos can pose as much of a threat in embryo transfer as actively infected embryos. Wrathall and Mengling (10,11) established that porcine parvovirus was unable to penetrate the zona pellucida and infect early porcine embryos but that it did attach to the zona pellucida. When these embryos were then transferred, the virus on the zona infected the recipient pigs and subsequently resulted in the death of the majority of embryos.

If IBRV cannot penetrate the zona pellucida and has merely become attached to this structure, then the possibility exists that the virus can be removed and the embryos rendered "clean". When IBR-antiserum was used to "disinfect" embryos (Table 3), the results were variable. If, after washing, the embryos were treated for 1/2 hour with antiserum and complement, there was a reduction in the number of positive embryos compared with embryos not treated. However, a number of embryos still carried infectious virus. When the incubation period was increased to one hour, infectious virus was removed from all of the embryos. After culturing for 24 hours, microscopic assessment of the embryos showed that, compared with untreated controls, incubation in antiserum and complement had not interfered with in vitro embryonic development (Table 3).

When IBRV-exposed embryos were treated with trypsin for 1-2 minutes prior to culturing (Table 3), infectious virus was removed from all of the embryos. After trypsin treatment, microscopic assessment of the embryos showed no change in either the zona pellucida or the in vitro embryonic development of these embryos when they were cultured for 24 hours.
Since both trypsin and IBRV-antiserum can render IBRV-exposed embryos uninfected, it is most probable that IBRV cannot penetrate the zona pellucida but merely attaches to the surface of this structure. The trypsin method is much faster than the IBRV-antiserum method. In addition, the success of the antiserum treatment was found to be dependant on time, whereas the trypsin treatment removed virus from all of the embryos treated. For these reasons, trypsin would be the better method of "disinfecting" IBRV carrying embryos if pregnancies can be obtained after trypsin treatment.

The 31 eggs/embryos collected from IBRV-seropositive donors were all found to be negative and did not require trypsin treatment. However, it should be emphasized that these donors were IBRV-seropositive and not necessarily viremic at the time of collection.

To allow conclusion regarding the control of IBRV using embryo transfer, embryos from IBRV-viremic donors will have to be transferred to "clean" recipients and the resulting calves monitored. If the in vivo situation parallels the in vitro one, then trypsin treatment will be required prior to embryo transfer.
REFERENCES


