Preventing disease transmission through the transfer of in-vivo-derived bovine embryos

D.A. Stringfellow*, M.D. Givens

Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849-5519, USA

Abstract

Investigation and experience have demonstrated that movement of in-vivo-derived bovine embryos can be accomplished while effectively limiting spread of infectious disease between populations of cattle. Experimental and theoretical justifications of current strategies for production of specific-pathogen-free, in-vivo-derived embryos are reviewed. Hazards of spreading bovine viral diarrhea virus via in-vivo-derived embryos are dealt with specifically. It is concluded that established sanitary procedures for producing pathogen-free, in-vivo-derived embryos are efficacious if the ethical and technical excellence of those performing the procedures can be assured. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Bovine embryo; Specific-pathogen-free embryo; Bovine viral diarrhea virus

1. Introduction

Introduction of postnatal animals and semen were traditional methods used to replenish and improve bloodlines in populations of cattle. Then, in the 1970s, technological advances provided efficient methods for nonsurgical collection, cryopreservation and nonsurgical transfer of pre-implantation bovine embryos. Thereafter, an alternative with obvious economic and humane advantages was available for movement of germ plasm between populations of cattle.

When embryo transfer became an option, there was understandable concern that the technology might provide a new mode for transmission of disease. Early concerns about transfer of infectious agents with embryos were based on embryo–pathogen observations that had been made in laboratory animals (see review by Wrathall and Sutmoller, 1998). However, after consideration of epidemiologic factors associated with production and transfer of in-vivo-derived bovine embryos, it was hypothesized that embryo transfer in this species would prevent transmission of disease if appropriate precautions were taken. Subsequent research on specific bovine pathogens within the context of embryo production technology supported the hypothesis and provided a foundation for today’s safe-embryo-handling recommendations.

The aim of this paper is to review epidemiologic aspects of bovine embryo transfer and results of early research that provided the basis for sanitary guidelines found in the Manual of the International
Embryo Transfer Society. In addition, results of more recent research are summarized with emphasis on bovine viral diarrhea virus. Finally, we discuss the impact that results of more recent studies and accumulated experience with commercial embryo transfer have had on our current view of strategies for pathogen-free-embryo production.

2. Epidemiological view of embryo transfer

Details of agent, host and environmental factors which collectively support the use of embryo transfer as a method for controlling the spread of pathogens were reviewed previously (Stringfellow, 1985; Stringfellow and Wright, 1987; Stringfellow et al., 1991) and are only summarized here.

If a pathogen was to be transmitted by transfer of in-vivo-derived bovine embryos, an uninterrupted sequence of events would have to occur (Fig. 1). Key elements in this hypothetical chain of events include: (1) exposure of embryos to the pathogen, (2) continued association of pathogen with the embryos, (3) maintenance of infectivity of pathogen throughout embryo manipulation and processing, and finally, (4) delivery of an infective dose of pathogen to a susceptible recipient. It is evident that a variety of limiting factors work to prevent the occurrence of this complete sequence of events.

2.1. Factors limiting exposure of embryos to pathogens

Under ordinary conditions, numerous factors tend to restrict exposure of embryos to pathogens. Especially important are, limited mobility of embryos, limited distribution of pathogens and precautions implicit in or applied to management of donor animals.

Between conception and collection, embryos are restricted to the uterine tubes and uterus of the donor cow. Consequently, a cow that is uninfected serves as an isolation unit for the preimplantation embryo prior to collection. If herds or regions of origin of donor cattle are free from specific pathogens, the security of the isolation is enhanced. If a donor cow is infected with an agent of disease, it is still possible that embryos might remain unexposed. The classic example is a cow infected with *Brucella abortus*. While this bacterium is a reproductive pathogen, an accumulation of evidence from studies in the 1980s (see review by Stringfellow and Wright, 1989) indicated that exposure of preimplantation embryos in the uterus of an infected, superovulated cow is
highly unlikely. The primary reason is that brucella do not remain in the postpartum uterus after multiple estrous cycles and will only return to the uterus long after conception when the fetus and placenta are well developed.

Donor cattle, representing the highest genetic merit, are normally afforded the best available health care. The herds of origin are often free of many pathogens due to participation in disease-control programs and enforcement of judicious herd-replacement policies. The extent to which preventive management and medicine is practised can be evaluated by the veterinarian who is a member of an official embryo collection team (Evans, 1998). The team veterinarian also can clinically assess the health of the donor cow at or around the time of embryo collection. Finally, some national regulatory authorities may request specific tests of donor cattle to further enhance safety when embryos are to be moved internationally.

2.2. Factors limiting continued association of pathogens with embryos

If embryos were to be exposed to pathogens, several factors tend to prevent continued association. These include inherent resistance of the embryo provided by the zona pellucida and natural or prescribed cleansing associated with embryo collection and transfer.

The mammalian zona pellucida is a distinctive extracellular matrix that ensures species specificity of fertilization, block to polyspermy and protection of the embryo during very early stages of development (Dunbar, 1983). However, it is well known that the zona pellucida functions as a microporous membrane, allowing traffic of relatively large molecules (Sellens and Jenkins, 1975). This permeability and results of several early studies in laboratory animals led to concerns that the zona pellucida might not provide a significant barrier to pathogens. As examples, in one report, the passage of Mengo virus through the murine zona pellucida was demonstrated (Gwatkin, 1967), and in another report, the genetic spread of the retrovirus that caused murine mammary tumor was reported (Bentivelzen et al., 1970). However, it is important to note that the bovine zona pellucida is substantially thicker (up to 15 \( \mu \text{m} \)) (Riddell et al., 1993c; Betteridge, 1995) when compared to the murine zona pellucida (about 5 \( \mu \text{m} \)) (Gwatkin, 1967), and there is no current proof of true vertical transmission (via hereditary incorporation) of retroviruses or other pathogens in cattle. To illustrate the latter, a retrovirus of cattle, bovine leukemia virus, is not incorporated into the genome of gametes (Divers et al., 1995). Without concern for infectious disease transmission via the gametes, it was especially important to confirm that the relatively thick bovine zona pellucida could serve as an effective barrier. In various studies, summarized below, it was confirmed that pathogens would not penetrate this barrier, and only a few would adhere to it. Still, an important concern was that pathogens found in body fluids or as contaminants in media might remain in close proximity to the embryo until the time of transfer.

Techniques for collection, processing and transfer of in-vivo-derived embryos vary, but in each situation, there are dilution factors associated with the sheer volume of recovery and holding medium that would serve to dilute any pathogen that might be present in the embryo’s environment. Also, prescribed procedures for washing, with or without trypsin, ensure that certain pathogens will be eliminated by dilution, dislodging, or inactivation (Stringfellow, 1998). Finally, the use of antibiotics in media for recovery, culture and storage of embryos effectively deters the spread of some prokaryotic pathogens and suppresses nonpathogenic microbial contaminants (Riddell and Stringfellow, 1998).

2.3. Factors limiting infectivity and delivery of an infectious dose of pathogens to recipients

Media and techniques for collection, storage and transfer of bovine embryos are intended to ensure that embryos maintain their developmental competence, but they do not necessarily ensure that infectivity of associated pathogens is maintained. An example of a negative impact of embryo processing on viability of pathogen was illustrated in an early study in which a standard embryo-cryopreservation procedure resulted in 64% or 99.9% reduction in viability of \textit{Brucella abortus} in the absence or presence of antibiotics, respectively (Stringfellow et al., 1986).
The last link in the hypothetical chain of infection is delivery of an infectious dose of pathogen along with a viable embryo into the uterus of a susceptible recipient (Fig. 1). It should not be presumed that all recipients are susceptible via the intrauterine or any other route. Some may be resistant to infection based on naturally acquired immunity (through prior exposure) or induced immunity (through vaccination). Even if recipients are susceptible to infection with a specific pathogen, the amount of infectious agent that is associated with a single embryo may not constitute an infective dose. The natural and prescribed obstacles such as dilution factors and washing that were described above would all tend to reduce the inoculum. In reality, there has been no report of a comprehensive study to deal specifically with intrauterine, day 7 inoculations with any bovine pathogen.

3. Early research providing the basis for sanitary guidelines

By the end of the 1970s, embryos could be collected nonsurgically from superovulated cows, cryopreserved for extended periods and transferred to recipient cows resulting in viable pregnancy with a high degree of consistency. The potential usefulness of these procedures in international trade led to questions by regulatory authorities and scientists about the role of embryo transfer in transmission of infectious diseases. Consequently, embryo–pathogen interactions became an important topic of investigation.

Selection of infectious agents for study was somewhat serendipitous; but usually, the pathogens were either objects of national prevention, control and eradication efforts, or there was some reason to believe that embryos might be exposed to them. Most studies utilized one of four standard experimental approaches (Bielanski and Hare, 1998). In one of these approaches, zona pellucida intact embryos were exposed to pathogen in vitro, washed and then assayed in vitro to determine if infectious agent was present. In Table 1, collective results are presented for some initial studies of this type. As regards the studies summarized in this table, it is important to note that the artificial exposure generally was to high concentrations of pathogen to mimic a worse case scenario. Also, washing procedures generally conformed to a protocol that has since been adopted as the recommended procedure of the Inter-

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>No. of embryos exposed</th>
<th>Embryos positive for pathogen (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akabane virus</td>
<td>80</td>
<td>0</td>
<td>Singh et al., 1982a</td>
</tr>
<tr>
<td>Bovine herpesvirus-1</td>
<td>107</td>
<td>70–100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Singh et al., 1982b; Stringfellow et al., 1990</td>
</tr>
<tr>
<td>Bovine herpesvirus-4</td>
<td>29</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Stringfellow et al., 1990</td>
</tr>
<tr>
<td>Bovine leukemia virus</td>
<td>12</td>
<td>0</td>
<td>Hare et al., 1985</td>
</tr>
<tr>
<td>Blue tongue virus</td>
<td>120</td>
<td>0</td>
<td>Bowen et al., 1982; Singh et al., 1982a</td>
</tr>
<tr>
<td>Bovine viral diarrhea virus</td>
<td>122</td>
<td>0</td>
<td>Potter et al., 1984; Singh et al., 1982a</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus</td>
<td>169</td>
<td>0</td>
<td>Singh et al., 1986</td>
</tr>
<tr>
<td>Rinderpest virus</td>
<td>200&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1–2</td>
<td>Mebus, 1988</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>144</td>
<td>36–50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Lauerman et al., 1986; Singh and Thomas, 1987; Stringfellow et al., 1989</td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>96</td>
<td>0</td>
<td>Mallek et al., 1984; Stringfellow et al., 1984</td>
</tr>
<tr>
<td>Haemophilus somnus</td>
<td>38</td>
<td>26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Thomson et al., 1988</td>
</tr>
<tr>
<td>Mycoplasma bovis</td>
<td>111</td>
<td>100</td>
<td>Bielanski et al., 1989; Riddell et al., 1989</td>
</tr>
<tr>
<td>Mycoplasma bovigenitalium</td>
<td>49</td>
<td>100</td>
<td>Riddell et al., 1989</td>
</tr>
<tr>
<td>Mycobacterium paratuberculosis</td>
<td>20</td>
<td>30</td>
<td>Rohde et al., 1990</td>
</tr>
<tr>
<td>Ureaplasma diversum</td>
<td>26</td>
<td>100</td>
<td>Britton et al., 1988</td>
</tr>
</tbody>
</table>

<sup>a</sup> This table is adapted from tabulated material in Appendix B, Manual of the International Embryo Transfer Society (Anonymous, 1998b).

<sup>b</sup> Trypsin treatment was thought to be effective for removal of this pathogen.

<sup>c</sup> Antibiotic treatment was thought to be effective for removal of this pathogen.
Table 2

Summary of results of early studies in which zona-pellucida-intact bovine embryos from infected or seropositive donor cows were washed and assayed for the pathogen

<table>
<thead>
<tr>
<th>History of donor cow</th>
<th>No. embryos (ova) collected</th>
<th>Assay of embryos/ova</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine leukemia virus-seropositive</td>
<td>60 (26)</td>
<td>Negative</td>
<td>Bouillant et al., 1981</td>
</tr>
<tr>
<td>Bovine viral diarrhea virus-seropositive</td>
<td>2 (21)</td>
<td>Negative</td>
<td>Singh et al., 1982a</td>
</tr>
<tr>
<td>Bluetongue virus-infected</td>
<td>51 (14)</td>
<td>Negative</td>
<td>Bowen et al., 1983</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus-infected</td>
<td>372 (64)</td>
<td>Negative</td>
<td>McVicar et al., 1987; Mebus and Singh, 1988</td>
</tr>
<tr>
<td>Bovine herpesvirus-1-seropositive</td>
<td>13 (18)</td>
<td>Negative</td>
<td>Singh et al., 1982b</td>
</tr>
<tr>
<td>Bovine herpesvirus-1-infected</td>
<td>63b</td>
<td>Negative</td>
<td>Singh et al., 1983</td>
</tr>
<tr>
<td>Rinderpest virus-infected</td>
<td>107 (embryos and ova combined)</td>
<td>Negative</td>
<td>Mebus, 1987</td>
</tr>
<tr>
<td>Brucella abortus-infected/seropositive</td>
<td>309 (82)</td>
<td>Negative</td>
<td>Barrios et al., 1988; Stringfellow et al., 1988; Voekel et al., 1983</td>
</tr>
<tr>
<td>Chlamydia psittaci-infected</td>
<td>5</td>
<td>Negative</td>
<td>Bowen et al., 1978</td>
</tr>
</tbody>
</table>

*a* This table is adapted from tabulated material in Appendix B, Manual of the International Embryo Transfer Society (Anonymous, 1998b).

*b* These embryos were treated with trypsin as part of the washing procedure prior to assay.

national Embryo Transfer Society (Stringfellow, 1998). For five of the nine viral pathogens (aka bane virus, bovine leukemia virus, bluetongue virus, bovine viral diarrhea virus and foot-and-mouth disease virus), embryos were free of infectious agent after artificial exposure and washing. For three other viral pathogens (bovine herpesvirus-1, bovine herpesvirus-4, and vesicular stomatitis virus) washing was not totally effective, but washing with trypsin was considered to be effective for producing virus-free embryos. The report that rinderpest virus was isolated from a small proportion (1–2%) of embryos after artificial exposure and washing was a preliminary report that was never confirmed, and it was later shown that zona-pellucida-intact embryos and ova collected from cattle infected with rinderpest were free of virus after washing (Table 2). Thus, experimental evidence had accumulated to indicate that viral pathogens were not likely to penetrate the bovine zona pellucida, and the few that adhered to the zona pellucida could be effectively removed by washing with trypsin.

Likewise, applied treatments were effective for removal of the bacterial pathogens listed in Table 1. Brucella abortus was effectively removed by washing and Haemophilus somnus was sensitive to treatment with antibiotics. The remaining mycoplasmal and mycobacterial pathogens were not removed by any treatment, after artificial exposure, but the potential for natural exposure of embryos to these agents with subsequent carriage from the in vivo environment (donor cow) has not been shown.

To determine if pathogens might associate differently with embryos if exposure occurred in vivo, a number of studies were conducted in which zona-pellucida-intact bovine embryos or ova and uterine recovery media from infected or seropositive donor cows were assayed for pathogen. The collective results of these studies were that pathogen could often be found in the recovery media when donors were known to be infected (Table 3), but after proper washing or trypsin treatment, pathogen was never isolated from embryos or ova (Table 2).

Finally, more expensive studies and field trials were conducted in which embryos were collected from infected or seropositive donor cattle and transferred to uninfected recipients. Afterward, the recipients and offspring were monitored for infection. For the six viral and one bacterial pathogen evaluated in this way, no recipient or calf sero-converted (Table 4).

4. Impact of the early research on approaches to health certification of embryos

Three strategies that have been used to produce specific-pathogen-free embryos are testing of donor cattle, embryo treatment or a combination of donor testing and embryo treatment (Wrathall and Sutmol-
Table 3
Summary of results of early studies in which uterine recovery media from infected or seropositive donor cows were assayed for the pathogen

<table>
<thead>
<tr>
<th>History of donor cow</th>
<th>Uterine recovery medium (no. positive/total)</th>
<th>Percent positive</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine leukemia virus-seropositive</td>
<td>4/25</td>
<td>16%</td>
<td>Bouillant et al., 1981</td>
</tr>
<tr>
<td>Bluetongue virus-infected</td>
<td>12/30</td>
<td>40%</td>
<td>Bowen et al., 1983;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thomas et al., 1985</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus-infected</td>
<td>15/22</td>
<td>68%</td>
<td>McVicar et al., 1987;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mebus and Singh, 1988</td>
</tr>
<tr>
<td>Bovine herpesvirus-1-infected</td>
<td>9/33</td>
<td>27%</td>
<td>Singh et al., 1983</td>
</tr>
<tr>
<td><em>Brucella abortus</em>-infected/seropositive</td>
<td>9/116</td>
<td>8%</td>
<td>Stringfellow et al., 1982,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1983, 1988; V oekel et al., 1983</td>
</tr>
</tbody>
</table>

* This table is adapted from tabulated material in Appendix B, Manual of the International Embryo Transfer Society (Anonymous, 1998b).

Table 4
Summary of results of early studies in which embryos were transferred from infected or seropositive donor cows

<table>
<thead>
<tr>
<th>History of donor cows</th>
<th>No. of embryos transferred</th>
<th>Serologic status of recipient/offspring after transfer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine leukemia virus-seropositive</td>
<td>596</td>
<td>Negative</td>
<td>Eaglesome et al., 1982;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DiGiacomo et al., 1986;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kaja et al., 1984;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hare et al., 1985;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Olson et al., 1982;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Parodi et al., 1983</td>
</tr>
<tr>
<td>Bovine leukemia virus-seropositive</td>
<td>&gt;1500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Negative</td>
<td>Thibier and Nibart, 1987</td>
</tr>
<tr>
<td>Bluetongue virus-infected</td>
<td>334</td>
<td>Negative</td>
<td>Bowen et al., 1983;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acree, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thomas et al., 1983, 1985</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus-infected</td>
<td>149</td>
<td>Negative</td>
<td>Mebus and Singh, 1988</td>
</tr>
<tr>
<td>Bovine herpesvirus-1-infected</td>
<td>64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Negative</td>
<td>Singh et al., 1983</td>
</tr>
<tr>
<td><em>Brucella abortus</em>-infected/seropositive</td>
<td>&gt;1500&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Negative</td>
<td>Thibier and Nibart, 1987</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>Negative</td>
<td>Del Campo et al., 1987</td>
</tr>
</tbody>
</table>

<sup>a</sup> This table is adapted from tabulated material in Appendix B, Manual of the International Embryo Transfer Society (Anonymous, 1998b).
<sup>b</sup> Embryos were cycling/superovulated cows.
<sup>c</sup> Embryos were collected from donor herds in which most of the cows were bovine leukemia virus- and bovine herpesvirus-1-seropositive.
<sup>d</sup> Embryos treated with trypsin between collection and transfer.

The cautious approach of donor testing was applied initially to document the health of embryos that moved between countries. However, by the mid 1980s much of the early research (Tables 1–4) had been completed and published. It had become clear that risks of transmission of diseases by embryo transfer were low, and the value of embryo treatments that had been used so effectively in research was recognized. Accordingly, the research protocols for washing and trypsin treatment were standardized by the International Embryo Transfer Society (Stringfellow, 1998) and the value of their use was conveyed by the Office of International Epizooties in their International Animal Health Code (Anonymous, 1994).

Standardized embryo washing and trypsin treatments are detailed in the Manual of the International Embryo Transfer Society (Stringfellow, 1998). Briefly, essential requirements for embryo washing are as follows: only groups of 10 or fewer, zona-pellucida-intact embryos from a single donor should be washed together. A minimum of 10 washes are applied, using separate sterile micropipets between each two washes such that each wash is a 100-fold dilution of the previous wash. After washing, embryos should be free of observable adherent material and free of breaks in the zona pellucida when viewed...
at 50 × magnification. Trypsin treatment has the same general requirements as washing except that there are 12 washes. Embryos are pre-washed five times, exposed to trypsin in a 6th and 7th wash for a total of 60 to 90 s and then washed five more times without trypsin.

Depending on circumstances, donor testing might still be used in conjunction with or in lieu of embryo treatment to certify health of transported embryos (Anonymous, 1994). However, it is noteworthy that the Research Subcommittee of the IETS has concluded that sufficient evidence has accrued to show that the risk of transmission of certain diseases is negligible if embryos are properly treated between collection and transfer. These diseases are bluetongue, Brucella abortus, enzootic bovine leukosis, foot-and-mouth disease, and infectious bovine rhinotracheitis (IETS to the OIE; Anonymous, 1998a).

5. Embryo–pathogen research in the 1990s

The emphasis of embryo–pathogen research in the 1990s has shifted to deal more with the newer in-vitro-derived embryo technologies. However, there have been some additional reports with relevance to in-vivo-derived bovine embryo production, and some questions still remain to be answered.

5.1. Additional ‘in vitro exposure and in vitro assay’ studies with bovine viruses

In a report by Gillespie et al. (1990), the infective status of bovine embryos after artificial exposure to seven viruses was described with results seeming to contradict those in previous studies. They declared that pseudorabies virus, bovine herpesvirus-1, vesicular stomatitis virus, bluetongue virus, and bovine viral diarrhea virus all adhered to the bovine zona pellucida after in vitro exposure but that bovine enterovirus and parainfluenza-3 virus did not adhere. Unfortunately, embryos were either not washed or washed only five times between exposure and assay, indicating that their methodology may have been responsible for results that conflicted with those in previous reports. For example, in early studies bluetongue virus and bovine viral diarrhea virus did not associate with zona-pellucida-intact embryos after in vitro exposure when the 10-wash protocol described above was used (Bowen et al., 1982; Singh et al., 1982a). In comparing this 1990 report to previous reports, the logical conclusion would seem to be that inadequate washing allows a greater potential for the transmission of disease with embryos.

The reputed value of the 10-wash protocol for ensuring freedom from foot-and-mouth disease virus was reaffirmed in a report by Camaano et al. (1993). In their study, 94 zona-pellucida-intact bovine embryos and ova were exposed to high titers (10^7.5) of foot-and-mouth disease virus for 16 h and washed as recommended in the IETS protocol. Then 79 embryos/ova were assayed immediately for infectious virus while 15 embryos were cultured for 24 h before testing. No virus was isolated from any of the properly washed embryos/ova, and exposure to the virus had no apparent effect on embryonic development in the cultured group.

5.2. Testing novel treatments for bovine embryos after artificial exposure to pathogens

Since early studies identified prokaryotic pathogens that adhered to the zona pellucida after artificial exposure and washing or trypsin treatment (e.g. mycoplasmas; Britton et al., 1988; Bielanski et al., 1989; Riddell et al., 1989) there has been only slight progress towards development of effective treatments to deal with these pathogens. Use of antibiotics in embryo culture and wash media has continued to be based on conventional recommendations for their use in cell culture media (Riddell and Stringfellow, 1998), but some insight has been gained into how antibiotics could be applied to combat prokaryotes. Increasing the concentration and time of exposure to currently used antibiotics as well as increasing the temperature of treatment media have all been tried.

Riddell et al. (1993a) reported that treatment with kanamycin (1000 µg/ml) or tylosin (200 µg/ml) was effective for producing Mycoplasma bovis-free bovine embryos when the artificially exposed embryos were washed 10 times and then incubated (37°C) for an additional 4 h in media containing the antibiotic. The concentrations of antibiotics in these treatments were higher than recommended by their
suppliers, but treatments had no apparent detrimental effects on the embryos. In a similar study, Otoi et al. (1993) demonstrated that bacteria-free bovine embryos could be produced after artificial exposure to Escherichia coli if they were incubated (38.5°C) for 2 h in medium with gentamicin (50 μg/ml) prior to washing 10 times. In an attempt to develop a novel treatment, Riddell et al. (1993b) tried to use organic halamines to inactivate Mycoplasma bovis adhering to bovine embryos. The mycoplasma were effectively inactivated, but there was insufficient margin for error between mycoplasmacidal and embryocidal concentrations to recommend regular use of these chemicals.

In a novel approach for inactivation of viruses associated with embryos, Bielanski et al. (1992) successfully used photosensitive agents (hematoporphrin and hematoporphrin derivative) to inactivate bovine herpesvirus-1 and bovine viral diarrhea virus. Their treatment had no apparent negative effect on the viability of embryos, but there is a need to determine if normal rates of pregnancy with birth of normal calves could be achieved after transfer of embryos treated in this way.

Certainly, added security could be provided by new broad-spectrum treatments for embryos, but there appears to be little current interest in research in this area. Presumably, this is because current protocols for certifying the health of embryos have been efficient and effective.

5.3. Collection and assay of embryos from infected donor cows or transfer of embryos after in vitro exposure to pathogen

In additional investigations in the 1990s, embryos were collected from donors that were artificially or naturally infected with bovine leukemia virus, bluetongue virus, foot-and-mouth disease virus or bovine spongiform encephalopathy. Also, in one study, embryos were subjected to long term exposure in vitro to bluetongue virus. Then, as in earlier studies, the embryos were evaluated for their potential to transmit the pathogens by direct assay in vitro or by transfer to recipients with subsequent evaluation of recipients and offspring for pathogen.

Krolinski et al. (1994) evaluated a four-step, embryo wash procedure to ensure that lymphoid cells were effectively removed when embryos were collected from bovine leukemia-infected donor cows. Subsequently, 585 embryos collected from naturally infected donors were washed four times and transferred, resulting in 278 pregnancies. Four calves dying at parturition were not tested, but the other 274 were free of bovine leukemia virus.

In a small study, Schlafer et al. (1990) incubated day 6 embryos in bluetongue virus-infected cell cultures for 24 h, washed them three times and then transferred two embryos into each of three seronegative recipients. None of the three heifers became pregnant, but virus was isolated from blood and a vaginal swab from one of the three heifers taken on the 7th day after the embryos were transferred. Because of the experimental procedures used, it is impossible to determine if adherence of the virus to embryos or inadequate washing was responsible for transmission of the virus.

In a large scale study reported by Acree et al. (1991), 60 heifers were artificially exposed to bluetongue virus. Embryos were collected from 39 of these heifers during either the acute or convalescent stage of the disease. Embryos also were collected from serologically positive donors that were presumed to have been naturally infected during the previous year. Thus, they were considered to be recovered donors. A total of 169, 141 and 52 embryos were collected from acute, convalescent and recovered donors, respectively, and washed 10 times according to the IETS standard procedure. In vitro assays (cell culture and embryonating chicken eggs) of 57, 20 and 25 of the embryos, respectively, were negative for bluetongue virus. Furthermore, 248 embryos (110 from acute, 121 from convalescent and 17 from recovered donors) were transferred to seronegative recipients. A total of 95 calves were born (36 from acute, 52 from convalescent and seven from recovered donors). There was no evidence of transmission of bluetongue to any recipients (or offspring) with embryos from acute or convalescent stage donors. However, two recipients of embryos from recovered donors (and subsequently their offspring) seroconverted between the 5th and 9th month after transfer of embryos. Subsequent investigation yielded evidence that these recipients were naturally exposed to bluetongue virus in mid to late pregnancy due to a breach in isolation of recipients. Failure to
adequately protect the latter recipients from insect vectors is unfortunate, but failure to transmit virus with embryos from all of the acutely and convalescently infected donors is still a strong endorsement for the IETS washing protocol.

There were two reports of studies evaluating potential for use of embryo transfer to salvage genetic material from foot-and-mouth virus-infected cattle. Villar et al. (1990) reported on a field trial in Argentina in which 253 embryos were recovered from 48 foot-and-mouth virus seropositive (convalescent) cows and washed according to IETS procedures. The flushing fluids and 171 of the embryos were tested for presence of virus by isolation in cell culture or intradermal lingual inoculation of negative cattle. All results were negative. In addition, the remaining 82 embryos were cryopreserved and 42 were transferred to seronegative recipients in a foot-and-mouth-disease-free area of the country. Fourteen live calves were born. The calves and recipients remained seronegative. Thus, the washing procedure and embryo transfer procedures were confirmed to be useful for preservation of germ plasm from infected populations of cattle.

Mebus and Singh (1991) reported results of a comprehensive evaluation of infective potential of embryos collected from donors acutely infected with foot-and-mouth disease virus. In this study, 436 embryos/ova were collected at slaughter from 30 superovulated cows that had been exposed by intravenous inoculation of foot-and-mouth disease virus 22 h prior to collection of embryos. All except two of the donors had a detectable viremia at the time of embryo collection, and virus was isolated from the uterine recovery medium from seven donors. All embryos and ova were washed according to IETS protocols prior to assay, cryopreservation or transfer. Two hundred and four washed embryos and ova were sonicated and injected (via intradermal lingual route) into steers that remained clinically normal and seronegative. Thirty-two embryos/ova with defects in the zona pellucida were assayed in cell culture and no virus was isolated. One hundred and six fresh embryos and 43 cryopreserved embryos were transferred to 80 and 31 recipients, respectively. All recipients remained seronegative and clinically normal. The outcome of these transfers were 15 normal calves, five sets of twins dead after premature birth and two fetuses aborted at 5 months of gestation. Sera from normal calves and one set of premature twins were negative for anti-foot-and-mouth-disease virus antibody. Thus, collective results showed that the IETS protocol for washing was effective for producing embryos without detectable amounts of associated virus regardless of whether in vitro or in vivo assays were used.

Finally, the enormous task of determining if properly washed embryos from donor cows with clinical bovine spongiform encephalopathy (BSE) might carry the disease to recipients or their embryo transfer offspring was begun in 1990. Because of the long incubation period of the disease, this study is not scheduled to conclude until the year 2001. A current update of results is found elsewhere in this issue (see BSE update by Wrathall). Preliminary progress reported by (Wrathall et al., 1997) indicated that bioassays of degenerate embryos/ova and uterine recovery medium from BSE affected cows were all negative.

6. Bovine viral diarrhea virus (BVDV)

Bovine viral diarrhea virus is an economically significant pathogen that has worldwide distribution among populations of cattle (Baker, 1995). The virus is known to be associated with semen (Guerin et al., 1992), ovaries (Booth et al., 1992) and serum (Brock, 1998) from infected cattle. Programs to eradicate the virus in cattle have been considered in several countries. In two early studies (Singh et al., 1982a; Potter et al., 1984) zona pellucida-intact, in-vivo-derived, bovine embryos were reported to be free of virus after artificial exposure to cytopathic isolates of BVDV, washing and in vitro assay for virus (Table 1). Several more recent studies have confirmed the value of IETS treatments for producing BVDV-free embryos. However, results of other research have created some doubt.

6.1. Association of BVDV with oocytes

While it has been known for some time that noncytopathic BVDV can result in early embryonic death or be transmitted vertically from cow to fetus resulting in the birth of a persistently infected calf
(Brownlie, 1990), presence of pathogens within the female gamete had not been considered a serious concern relative to in-vivo-derived embryo production in cattle. Recently, evidence for presence of BVDV in oocytes of developing follicles of cattle was reported by Fray et al. (1998). Their study was designed to investigate the cellular tropism of BVDV in the ovaries of three persistently infected heifers. Ovaries were collected from these heifers at post mortem and assessed for presence (by virus isolation) and localization (by immunohistochemistry) of BVDV. Samples from each ovary contained $10^7$ tissue culture infective doses/ml of BVDV. Immunofluorescence observed in cryosections of each ovary indicated the presence of viral proteins in ovarian stroma, thecal cells in the walls of developing follicles, cumulus cells and in a percentage of the oocytes examined. Approximately 2000 oocytes were viewed in sections from all of the ovaries with the observation that about 18.7% contained viral antigen. Further, there was no difference in percentage of BVDV-infected oocytes between those in primordial (18.2%), primary (19.4%) and secondary (21.2%) follicles. While their conclusion was that oocytes and cumulus in developing follicles of persistently infected cows are infected with BVDV, the epidemiologically relevant question that remains unanswered is: are the infected oocytes developmentally competent?

In two similar studies, immunohistochemical techniques were used in an attempt to identify ovarian cell types that might be infected with BVDV following acute infection (Grooms et al., 1998a) and following immunization with modified live BVDV vaccine (Grooms et al., 1998b). Viral antigen was detected between 6 and 60 days after acute infections in interstitial stroma cells and macrophage-like cells that were associated with primary follicles, secondary follicles, antral follicles, corpus luteum and corpus albicans. However, specific staining of luteal, thecal or granulosa cells was not observed. After administration of modified live BVDV vaccines, viral antigen was detected in ovarian sections taken on days 10, 20 and 30 after vaccination. Again stained cells were stromal and macrophage-like cells in the ovarian cortex. The authors concluded in each study that the observed changes could lead to reduced fertility.

Comparing the results of those studies with persistently and acutely infected animals, it is tempting to speculate that oocytes are less likely to contain BVDV in acutely infected animals; nevertheless, oocyte infection in acutely infected animals cannot be discounted. Besides, regardless of the type of infection, we now have information that the female gamete could be infected with BVDV and the relevant epidemiologic question remains: are infected oocytes developmentally competent?

### 6.2. Developmental competence of oocytes from BVDV-infected cows

Whether oocytes containing BVDV are capable of final maturation, participation in conception and development to transferrable stage embryos is a question that remains to be answered, yet a little insight into the potential for this occurring is provided in some reports. For example, a negative impact of BVDV infection on ovarian function has been demonstrated. In a controlled study by Grooms et al. (1998c), acute infection with BVDV was associated with a reduction in maximum diameter and rate of growth of anovulatory and ovulatory dominate follicles as well as a reduction in the number of subordinate follicles.

Other investigations within the context of normal embryo production also demonstrated that BVDV infections reduced reproductive efficiency. The effect of acute infections on superovulatory responses was examined by Kafi et al. (1997). In their trial, approximately equal groups of uninfected ($n = 12$) and acutely infected ($n = 13$) Friesian heifers were treated for superovulation, observed for estrus, inseminated, and underwent nonsurgical embryo recovery. The infected heifers were inoculated intranasally with virus on the 9th day preceding the planned day of insemination. Only three of 13 infected heifers displayed signs of estrus compared to 10 of 12 control heifers. Also, the mean numbers of transferrable embryos from control and infected animals were 4.0 and 0.2, respectively.

In another report by Brock et al. (1997) the reproductive inefficiency of seven persistently infected donors was evident by their consistent failure to respond to superstimulation and by their consistent failure to conceive. A small number of transferrable
embryos was produced from these persistently infec-
ted heifers. Most of these embryos and nonfertilized
and degenerated ova were washed according to IETS
guidelines and found to be free of BVDV as de-
termined by virus isolation and PCR assay. Also, a
BVDV-free calf was born after transfer of an embryo
from one of the persistently infected donors to a
seronegative donor.

Two other reports have described the birth of
BVDV-free calves after transfer of embryos from
persistently infected donors. Wentink et al. (1991)
reported the collection of one viable and five degen-
erated embryos from a persistently infected cow.
Bovine viral diarrhea virus was isolated from re-
covery medium. The viable embryo was washed 10
times, treated with trypsin and transferred to a
BVDV-immune recipient, resulting in the birth of a
BVDV-free calf. Similarly, Bak et al. (1992)
superovulated and inseminated two persistently in-
fected heifers and recovered embryos nonsurgically
from the uterus. Uterine recovery medium from both
animals contained BVDV. Eight embryos were re-
covered from these two heifers and washed 10 times.
Then six embryos were transferred to six recipients.
Four recipients were pregnant at 35 days and two
calved. Both calves were free of BVDV.

The only evidence that BVDV might be associated
with transferrable embryos from persistently infected
cows was reported by Tsuboi and Imada (1998).
They superstimulated, artificially inseminated, and
nonsurgically collected embryos from persistently
infected Friesian heifers. A total of eight normal
embryos and four degenerated ova were recovered in
two collections from the first heifer while five
degenerated ova were recovered in a single collec-
tion from the second heifer. The zona-pellucida-
intact embryos and ova were washed three times and
frozen prior to RNA extraction and assay by RT-
PCR. Of 17 ova and embryos, BVDV RNA was
detected in association with one compact morula and
one blastocyst from the first heifer. Results of this
study would have had greater significance if the
embryos and ova had been washed using IETS
guidelines prior to the RT-PCR assay. As it is, the
virus-positive embryos might have been the result of
infections of the embryo, adherence of the virus to
the zona pellucida, or inadequate washing. It is
noteworthy that, as stated above, Brock et al. (1997)
failed to identify BVDV RNA with PCR assay after
ova were washed 10 times using the IETS protocol.

6.3. Conclusions about potential spread of BVDV
through transfer of in-vivo-derived embryos

Finding BVDV antigen in developing oocytes is a
concern, yet, to date, there is no report of BVDV
associated with zona pellucida-intact embryos that
have been washed according to the IETS protocol.
Further, the few calves (n = 4) produced from infec-
ted cows have been BVDV-free. Also, it is logical to
speculate that reduced reproductive efficiency of
infected cows might be partially due to incompetence
of oocytes that are infected, oocytes that are from
follicles with infected cells or oocytes from ovaries
that are impaired by stromal infection.

Of course, many questions remain to be answered.
Among other concerns, future research should ad-
dress (1) the competence of oocytes from infected
cows and (2) the efficiency of IETS washing pro-
cedures after in vitro exposure of embryos to repre-
sentative field isolates of type I and type II
noncytopathic BVDV.

7. Experience with natural disease transmission
via in-vivo-derived bovine embryos

To date there has been no documented case of an
infectious agent being transmitted through transfer of
an in-vivo-derived bovine embryo. This is despite
the enormous number of embryos that have been
transferred world wide over the past 20 years.
Statistics were not compiled until recently, so the
actual number of embryos transferred in the 1970s
and 1980s is not known. Some insight into 1980s
activity was provided by a survey conducted by the
IETS in which nearly 100,000 embryos were re-
portedly transferred in North America alone in 1986
(Kraemer, 1987). Since 1991, annual data have been
retrieved by the IETS through a worldwide survey of
the embryo transfer industry. According to the most
recent report 360,656 in-vivo-derived embryos were
transferred worldwide in 1997 (Thibier, 1998). The
statistics for 1991 to 1997 are illustrated in Fig. 2.

It is likely that tens of thousands, if not hundreds
of thousands of embryos were transferred before the
Fig. 2. Number of in-vivo-derived bovine embryos transferred worldwide (per IETS Data Retrieval Committee).

value of current washing and trypsin treatments were recognized and the techniques were consistently used. Worldwide implementation of the concept of approved embryo collection teams just began within the last few years (Evans, 1998). Thus, many of the embryos transferred, especially domestic transfers, in early years were performed without knowledge of the benefit of current sanitary procedures. Despite this circumstance, transmission of infectious agents was not observed. This early record of freedom from disease transmission with what might be considered inadequate precautions serves to reinforce the view of innate safety of the procedures. That many hundreds of thousands of embryos have been transferred annually in the 1990s without transmitting disease, supports the value of current sanitary protocols.

Of course, circumstances could change. It is conceivable that changes in technology, emerging pathogens, altered patterns of movement of embryos or some other factors might increase the potential for transmission of infectious agents through embryo transfer. Hence, it is important to maintain an awareness of changing technologies and their applications within the context of the health of our cattle populations. Continued conscientious application of our time-tested procedures for health certification of embryos is also important. Perhaps most critical to maintaining our current record of safety is the ethical and technical excellence of those who apply the technology of embryo transfer.

References


Stringfellow, D.A., Howell, V.L., Schnurrenberger, P.R., 1982. Investigations into the potential for embryo transfer from \textit{Brucella abortus} infected cows without transmission of infection. Theriogenology 18, 733–743.


