Anim. Reprod., v.12, n.3, p.444-449, Jul./Sept. 2015

Research challenges involving embryo pathogen interactions

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Abstract

In the late 1970s, embryo pathogen research was spawned. Initially, a great deal of funding was available to groups involved in embryo pathogen research. However, following years of research, and development of competent embryo processing procedures endorsed by the International Embryo Transfer Society (IETS), a growing belief developed that embryo transfer (ET) is innately safe and so funding for embryo pathogen research has since dwindled portentously. Even with continued growth of the ET industry and specifically with tremendous changes involving *in vitro* technologies, and recent outbreaks involving pathogens of concern, funding of ET research has not been a priority and/or a focus area for funding agencies for the last number of years. Funding issues are not the only challenge in embryo pathogen research but it is the primary challenge since no amount of research can be pursed without funding. Some of these additional challenges include; a large number and variety of pathogens which need to be systematically investigated including new and re-emerging pathogens, utilization of animal origin products which have the potential to harbor and transmit pathogens, the ability of pathogens to adapt and change to their hosts and environment resulting in variation of affinity and virulence, reliable testing of these pathogens and trained personnel to perform studies, collect and interpret data and to knowledgeably handle pathogens that have zoonotic pathogens. This paper reviews these challenges facing embryo pathogen research today.

Keywords: embryo pathogens, *in vitro* produced embryos, *in vivo* derived embryos.

Introduction

The techniques used for nonsurgical embryo recovery; including collection, cryopreservation, and nonsurgical transfer of *in vivo* derived (IVD) bovine embryos, rapidly evolved into a frequently utilized commercial procedure. Embryos then became available for international commerce. This created concerns of diseases, which might be transmitted inadvertently through embryo transfer (ET). It stimulated establishment of protocols to ensure removal and hence, prevention of pathogen adherence to embryos. Also, standardized regulations involving embryo movement were also constructed to further minimize any potential pathogen transmission. A tremendous amount of research has been completed to test these protocols and procedures. There is a great deal of confidence in the standard processing procedures which has in some cases has resulted in complacency in embryo pathogen research. However, more research is essential to ensure the safety of transfer of IVD embryos, *in vitro* produced (IVP) and cloned embryos as well. The objective of this paper is to discuss the current challenges involving embryo pathogen research.

Research development, regulations and funding

Since the late 1970s, the concern of inadvertent disease transmission via embryos and resultant infectious disease outbreaks following in suite spawned the need for research involving embryo pathogen interactions. Specifically, it was deemed that international movement of embryos needed to be regulated to prevent unwanted and foreign animal diseases from gaining entrance to countries currently listed as free from these regulatory diseases. In the 1970's through to the 1990's there was a significant amount of funding available for embryo pathogen research provided by government entities such as the United States Department of Agriculture and other regulatory bodies in other countries.

Initially there was an absence of uniform import requirements, which lead to importing countries developing expensive, often times cumbersome and sometimes arbitrary embryo-health certifying procedures. International regulations required herds and/or nations in which donors resided to be free of all diseases of potential concern to importing countries (Waters, 1981). Other regulations consisted of embryo donors being housed in isolation facilities for an acceptable period then to be tested and certified to be free from an assortment of infectious diseases. Additionally, the embryo recipient herds were required to be isolated throughout pregnancy in the country of destination and following birth they and their offspring where similarly tested to confirm absence of a variety of diseases. Hence, there was a tremendous need to develop sanitary collection and processing procedures, which could function as "broad spectrum" health certifying procedures. However, little research and data was available to determine what was necessary and little

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Received: May 14, 2015
Accepted: July 1, 2015

experimental evidence to confirm their beliefs (Atwell, 1987). This situation was producing an environment that would seriously undermine international movement of embryos. This state provided the stimulus necessary for gaining the funding to conduct essential research. Specifically, the research was needed to test the belief that zona pellucida-intact (ZP-I), IVD, bovine embryos were not likely to transmit infectious diseases if properly handled and processed even if some donors resided in "infected" countries.

Experimental approaches

The primary experimental hypotheses was that if the embryo had an intact zona pellucida and the embryos where properly handled it would protect the early conceptus from infectious agents and transmission of embryos would be minimal (Stringfellow *et al*., 2004; Givens *et al*., 2007). The null hypothesis was that diseases might be transmitted by transfer of embryos. It was determined in order to provide consistency in research so that data might be comparable, four main approaches where outlined and utilized (Bowen *et al*., 1978; Archbald *et al*., 1979; Hare, 1990). In the first approach, recovered ZP-I embryos from pathogen free donors were artificially exposed *in vitro* to a variety of pathogens that were of domestic or international concern. The embryos were subjected to cleaning protocols and then subsequently tested *in vitro* for the pathogen in question. The second approach was very similar with the exception that following experimentally exposure of the embryo to the pathogen or pathogens, the embryos were transferred to disease-free (sentinel) recipients. The recipients were subsequently observed and tested along with their offspring for presence of the agent. The third approach was to collect ZP-I embryos from infected donors, clean them, and subsequently, assay the embryos for pathogens. The fourth approach was to again collect the ZP-I embryos from infected donors and then transfer the cleansed embryos to disease-free (sentinel) recipients which along with their offspring were then observed and tested for the disease agents. The goal was that each pathogen of concern would undergo this comprehensive series of experiments using each of the four approaches. Hence, the data gathered from these studies involving the specific pathogens analyzed would be essentially conclusive and the likelihood of transmission of the pathogen via ET would be known.

Although, this plan seemed to be comprehensive there are shortcomings revolving around the reliability of the testing available. Also, the correct controlling of variables and the interpretation of the evidence presented within the population tested are always of concern. Although, there has been a decline in the number of new research studies involving embryos over the past decade, there has been a tremendous increase in number and type of technologies available for determining the presence of a pathogen or pathogens in culture systems. Additionally, the sensitivity of the available assays has also increased dramatically. The RT-q-PCR utilized in the above-mentioned studies is representative of the ability of current assays to determine the presence of specific disease agents to the point that the levels detected may be below what would actually constitute an infective dose. The development of numerous assays used for screening, detection, and quantification of specific pathogens has been prolific. Assays have been developed for a number of reasons including to be utilized in eradication programs and/or to be utilized as a research tool for those pathogens of greatest concern such as BVDV and BHV-19 (Marley *et al*., 2008; Gard *et al*., 2009; Gregg *et al*., 2009). However, these techniques require appropriate trained individuals with careful attention to detail in order to prevent false negative or false positive results leading to false conclusions.

Pathogens evaluated

A variety of pathogens has been studied, but there is a multitude which have not been, and more information is needed. A greater emphasis for study was placed on those of regulatory concern in either domestic or international commerce, including bluetongue virus, bovine spongiform encephalopathy, *Brucella abortus*, enzootic bovine leukosis virus, foot and mouth disease virus, and infectious bovine rhinotracheitis virus (bovine herpesvirus-1). Aujeszky's Disease in swine and the scrapie agent in sheep were also the objects of thorough study. The embryo washing procedures originally developed and validated in numerous studies provided the basis for the commonly accepted embryo processing procedures (a.k.a. embryo washing and trypsin treatment), which are recommended today for health certification of IVD embryos (Bowen *et al.*, 1979; Singh *et al.,* 1987; Thibier and Nibart, 1987; Stringfellow and Wright, 1989; Wrathall, *et al.*, 1995, Stringfellow, 2010).

Additionally, many of the pathogens which were evaluated early on were laboratory strains, which can be very different from wild strains. Certain pathogens mutate during replication on a regular basis, such as bovine viral diarrhea virus (BVDV), which mutates every time it replicates. Hence, the early experiments utilizing the laboratory strains did not evaluate a good spectrum of BVDV strains. Reports by Lindberg and Drew (Lindberg *et al*., 2000; Drew *et al*., 2002) stimulated further investigations to the possibility of transmission of BVDV via ET due to sero-conversion of heifers after embryo transfer and birth of a PI calf following ET, respectively. Contaminated Fetal Bovine Serum (FBS) was thought to be the inciting cause of these infections. Hence, additional studies were necessary to determine if different strains of BVDV would remain associated with BVDV following

standard IETS processing procedures for IVD embryos and whether this associated virus could be transmitted via ET. Studies were performed which highlighted the variation in affinity between different strains of BVDV and the affinity of these strains for embryos (Waldrop *et al*., 2004a, b). Some strains were shown to maintain association with embryos following IETS processing procedures including when trypsin treatment was added to the processing procedures (Waldrop *et al*., 2004a, b). These high affinity strains became more of a concern when it was determined that the embryo-associated virus was indeed infective in both *in vitro* and *in vivo* studies (Waldrop *et al*., 2004b, 2005). Current research by Gard *et al*. (2009) reported that 27% of IVD and 42% of IVP embryos had embryo-associated virus (EAV) following artificial exposure to a high affinity strain of BVDV (SD-1, type 1a) following washing procedures in accordance with IETS (without trypsin). It was also found that the range of the amount of EAV was 100 to 450 cell culture infective doses to the 50% endpoint ($CCID_{50}$)/embryo (Gard *et al*., 2009). In previous studies, EAV was also determined to be infectious in an *in vitro* culture system and in an *in vivo* model and through intravenous inoculation of embryos and then in an intrauterine inoculation of embryos and 898 ($CCID_{50}$) of BVDV (SD-1, type 1a) into virus negative and seronegative recipients (Waldrop *et al*., 2004b, 2005; Gard *et al*., 2009, 2010). It was found that all recipients of embryos and virus became viremic, and then seroconverted (Waldrop *et al*., 2005; Gard *et al*., 2009, 2010).

The finding that no BVDV positive offspring were produced in these studies is similar to the results found Bielanski *et al*. (1998). In this study *in vitro*-produced embryos were exposed to noncytopathic biotypes of BVDV for 1 h, type 2 strain (P-131) or a type 1 strain (NY-1), and then washed in accordance with IETS guidelines (no trypsin treatment) and then transferred to seronegative BVDV negative recipients. However, none of the recipients following intrauterine transfer of embryos exposed to the type 1 strain seroconverted but of the 35 recipients receiving embryos exposed to the type 2 strain 18 seroconverted (51%), and there were 11 pregnancies at 30 days post transfer but of these only two resulted in live offspring. These two offspring were determined to be BVDV negative and seronegative. Hence, the infection seemed not to be recognized by the fetus and/or the virus was destroyed prior to development of immune competence so in fact no antibodies were formed by the fetus. The results of no detectable seroconversion from the type 1 strain and abortion due to the type 2 BVDV may be the results of mutations within the virus, or the test applied might have had produced false negatives. In a study by Meyers *et al*. (2007), decreases in interferon production, abortion and presence of virus in fetal tissues did not result when pregnant cattle were injected with two different mutant strains of virus. Each of these mutant strains had mutations specifically affecting both the N-terminal protease (N(pro)) and the deletion of codon 349, which abrogates the RNAse activity of the structural glycoprotein E(rns; Meyers *et al*., 2007). However, decreases in interferon production, abortion and presence of virus in fetal tissues did occur with wildtype viruses and in viruses in which only one mutation of either N(pro) or E(rns) occurred (Meyers *et al*., 2007). Therefore, the establishment of persistent infections requires both N(pro) and E(rns). It is logical to ascertain that the type 1 strain utilized in Bielanski's study might have had mutations within these areas, resulting in adequate interferon production and no fetal infection. Each specific strain may affect interferon production differently and therefore may or may not result in fetal infection, and that fetal infection may or may not be fatal. A thorough evaluation is important as the ability to transmit BVDV via an embryo would necessitate re-evaluation of embryo health certification procedures along with implementation of additional regulations on embryos exported from BVDV-positive countries to those countries where BVDV has been eradicated. So, BVDV is a good example of challenges facing embryo pathogen research namely intra-pathogen mutations and variation.

Classical Scrapie and Atypical Scrapie pose another example of intra-pathogens variations, which may confound research findings when they are treated as the same. Studies have established that Classical Scrapie may be transmitted within and between flocks by various routes and that transmission when utilizing ET may occur (Detwiler and Baylis , 2003 Andréoletti *et al*., 2011). However, secondary cases involving Atypical Scrapie are rare (Fediaevsky *et al*., 2009, 2010; Garza *et al*., 2011) and a low transmissibility has been seen between sheep in case controlled studies (Fediaevsky *et al*., 2009, 2010; Garza *et al*., 2011) but further assessment are being performed (Hopp *et al*., 2006). Additionally, Atypical Scrapie is only detectable in brain tissue and not in lymphoreticular system so it is assumed to have a decreased risk of transmission via ET when compared to that of Classical scrapie (Wrathall *et al*., 2008; Garza *et al*., 2011; Ligios *et al*., 2011). Classical Scrapie has been detected in fetuses of scrapie-affected ewes (Nicholson *et al*., 2008; Garza *et al*., 2011) and semen from Scrapie-infected rams (Rubenstein *et al*., 2012) making the infection of embryos a significant possibility if these embryos are not processed in accordance with the IETS guidelines for IVD embryos (Stringfellow *et al*., 2010). However, if embryos are processed in accordance with IETS standards for IVD embryos than there would be a negligible chance for transmission involving Classical Scrapie in sheep, as it is a category 1 disease according to the OIE (Stringfellow *et al*., 2010). The OIE code chapter 4.7.14 (World Organisation for Animal Health - OIE *et al*., 2014a, b) does not includes Atypical Scrapie because it is known to be different than Classical

Scrapie. Hence, it has been suggested to the OIE by the HASAC subcommittee, that Atypical Scrapie should be described as a category 3 disease since more research is necessary to completely determine the risk of transmission even though it appears on evaluation to have less potential for transmission via embryos.

In vitro produced embryos

Furthermore, when evaluating *in vitro* produced (IVP) embryos verses IVD embryos there are clear differences such as differences in the zona pellucida, the potentially-contaminated materials of abattoir-origin that have been regularly utilized in IVF embryo production, and the multiple steps that might result in in advertent contamination (Stringfellow and Wrathall, 1995). Therefore, the potential to introduce bacterial and viral contaminants during the process of producing IVP embryos has been emphasized in many studies (Stringfellow Wrathall, 1995; Bielanski and Jordan**,** 1996; Givens *et al*., 1999, 2001, 2002; Gard *et al*., 2009). The more porous nature of the zona pellucida of IVP bovine embryos was evident by the results of those studying a number of viruses such as: bluetongue virus, bovine viral diarrhea virus, and foot and mouth disease. It was found that virus adhered to the ZP of the IVP embryos while they had not adhered to the ZP of the of the bovine IVD embryos (Marquant-Le Guienne *et al*., 1998; Stringfellow *et al*., 2004). It continues to be clear that embryo washing, while beneficial in reducing environmental pathogen load, would not be as universally reliable for certifying the health of IVP embryos and can result in confounding results in studies due to contamination. So, it is necessary that additional steps are taken when analyzing pathogens of IVP embryos such as: (1) establishing minimum standards for sanitation in the laboratory and handling of oocytes; (2) pre- testing of animals utilized for OPU and materials of animal origin for specific pathogens and contaminants; (3) the judicious use of antimicrobials; (4) continuous, follow-up testing for contaminants of samples from IVM, IVF and IVC cultures; (5) washing of oocytes and developed embryos using the techniques applied to IVD embryos as a complementary control measure to reduce environmental pathogen load; and (6) when possible utilize synthetic oviductal media to minimize contaminants in the system. Details of processing IVP and micromanipulated embryos to remove and prevent contamination are outlined by the OIE chapter 4.8, and 4.9. (OIE, 2014c, d).

Somatic cell nuclear transfer

The most recent embryo method for *in vitro* production of bovine embryos, identified as somatic cell nuclear transfer (SCNT) shares the same concerns that IVP embryos (Stringfellow *et al*., 2004), do except some added caveats exist. The first major concern would be the removal or at least fracture of the zona pellucida. Additional, avenues for contamination exist such as the long duration of cell culture lasting weeks to months allows for exponential chances of extraneous pathogens interfering within the system. Hence, strategies for health certification of resulting embryos have not focused on any specific methods for washing the embryos, but rather, they have focused on risks for introduction of infectious agents and testing protocols to certify that the embryos or materials of animal origin used in their production are specific-pathogen-free prior to transfer of the embryos or their movement in commerce (Stringfellow *et al*., 2004). This is imperative when utilizing SCNT embryos in studies so that variables can be managed within the study.

However, there is a continued false sense of security with IVD embryos, IVP embryos, as well as cloned embryos. The argument has been that since no major outbreaks have been traced back to ET, so, it must not be a viable problem. Hence, this security has resulted in complacency of funding agencies and embryo pathogen research is no longer listed as a focus area even in the face of recent outbreaks and studies which highlight a need for additional embryo pathogen research. Some believe that sufficient research has been completed and that even with increased international embryo movement, especially with IVP embryos, no steps should be taken to stimulate revenue for additional studies. This seems to be a short-sighted approach since IVP embryos are of particular concern because the standard processing procedures are not as affective at removal of pathogens. Also, evolution of pathogens is a constant process resulting in re-emerging pathogens and formation of new pathogens that have not been fully tested. In order for the ET industry to continue to have continued growth, additional research is necessary. There is a desperate need to increase research in embryo pathogen interactions to provide up to date information on the diseases of concern and to elevate commerce and the efficiency of the embryo production systems.

Zoonotic pathogens

Additionally, public health concerns can be raised with some pathogens, especially those that are zoonotic. A good example of this is seen with *Coxiella burnetti*, which has been detected in media from embryo collections and uterine tissue samples from goats (Alsaleha *et al*., 2013). In recent studies by Alsaleh *et al.* (2013, 2014*)*, they reported that *C. burnetti* was not removed when IVD caprine embryos and/or IVP bovine embryos were exposed *in vitro* and subsequently underwent standard washing procedures. The pathogen, *C. burnetti,* is zoonotic. Hence, handling of the donor and potential recipient contamination might result in human infection. Thus, appropriate handling of the donor, recipient and the embryos is necessary so as not

to institute disease in the researchers themselves.

Summary

There are many areas that are challenging to embryo pathogen research. The most primary is lack of funding. Since research requires funding, it is a guarantee that studies cannot be performed without it. Today, funding agencies do not list embryo pathogen research as a focus area even in the face of recent outbreaks and studies which highlight a need for additional embryo pathogen research. Embryo movement in commerce will be hampered if further research is not performed. Research is the key to increase the safety and efficacy of the embryo transfer industry. Additional challenges include; the large number and variety of pathogens, new and re-emerging pathogens, utilization of animal origin products, which often harbor and transmit pathogens, adaption of pathogen to their hosts and environment resulting in variation of affinity and virulence, reliable testing of these pathogens and trained personnel to perform studies, collect and interpret data, international regulations and the zoonotic potential of some pathogens.

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