REPRODUCTIVE BIOTECHNOLGIES AND RISKS OF DISEASE SPREADING

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SUMMARY

The present paper deals with the risk analysis of the three first generations of Reproductive Biotechnologies: Artificial Insemination (AI), in vivo collected and in vitro produced Embryo Transfer (ET). Those technologies are used at large and worldwide. There are theoretical risks to associate pathogens with gametes and embryos to be moved hence contaminating the recipients. For each of those technologies, as they were becoming available and used on the field, the veterinary community has been able to perform numerous investigations allowing to generate guidelines and recommendations based on sound science. The Intergovernmental Agency, the Office International des Epizooties (OIE) has assessed those elements and has approved those recommendations as now published in the Terrestrial Animal Health Code. For artificial insemination, the basic rules rely on the presence of semen donors in pathogen free studs under the official veterinary supervision. For transfers of both in vivo collected and in vitro produced embryos, and in addition to the strict application of the guidelines such as those published in the International Embryo Transfer Society (IETS) Manual, the basic concept of biosecurity relies on the officially approved embryo collection or production and transfer teams. Many decades have proven that when such guidelines and recommendations are rigorously followed, those transfers can be achieved with a maximum level of security.

INTRODUCTION

There are classically four generations of Reproductive Biotechnologies (RB) that have built up progressively (Thibier, 1990). The first introduced after the last world war is that of Artificial Insemination (AI) and the last, transgenesis appeared with the first breakthrough reported by Palmiter et al., (1982) in producing transgenic mice. In between, were those of "classical" Embryo Transfer (E T), involving in vivo embryo collection and transfer (second generation) and those of in vitro embryo transfer and nuclear transfer (cloning). Currently, on the field, it is a fact that only Artificial Insemination, in vivo collected embryos and in vitro produced embryos transfers are used with quite a different range in numbers. Insemination by artificial means has been developed in many species of mammals, birds and insects. A recent worldwide survey by Thibier and Wagner (2000, 2001) has shown that, in the bovine where it is the most widely applied, more than 100 million females are inseminated each year. This corresponds to approximately one sixth of the total population. It is now over a quarter of century since the second generation of RB became operational in the field. Due to the relatively high cost of obtaining offspring (around 7OO €, for cattle in the European Union), its global uptake has been restricted mainly to cattle. As shown by the annual survey of the International Embryo Transfer Society (IETS) Data Retrieval Committee, around 500,000 bovine embryos are transferred annually across the world (Thibier, 2003). The transfer of in vitro produced embryos became operative for special purposes in cattle and, to a lesser extent, in other species some ten years ago. More than 80,000 bovine in vitro produced embryos were transferred worldwide in 2002 (Thibier, 2003).

Since the beginning of implementation on the field of reproductive biotechnologies, starting with Artificial Insemination, in the late forties, the veterinary community has taken the greatest care to ensure that no pathogen transmission would be associated with semen. The same held true with the more recent reproductive biotechnologies.

The risk analysis, as called nowadays, includes first an assessment of the risks that some pathogens could be associated with the gametes or embryos that are collected or produced and further transferred. A considerable number of investigations have been made and have resulted into some recommendations based on sound science that were then taken and approved by the Office International des Epizooties (OIE) and further incorporated in the relevant OIE Appendices. We will here report in the first section of this presentation some highlights of this assessment. Risk management is the second step of the risk analysis and involves a clear, well-defined code of practice ensuring that the recommendations are followed all along the line of the process so as to guarantee transfer of pathogen free gametes or embryos. This will be here reported in the second section of this paper.

1. THE RISK ASSESSMENT.

Genital shedding of pathogens can result from primary infection of the genitalia or from a more generalized infection. It is hence quite logical to assume that there are risks of contamination associated with collecting gametes or embryos and transferring them in any recipient without appropriate control measures.

1.1. Semen sterility is virtually unachievable, so the first question is how to control the population of so-called "non-specific microorganisms in semen efficiently and the second question is how to prevent any association of specific pathogens.

Numerous studies have identified specific putative agents associated with semen that are able to contaminate inseminated cows (see reviews by Thibier, 1998 and Thibier and Guérin, 2000). Are so concerned the 15 major diseases listed by OIE in List A (table 1). They are all of viral origin with the exception of contagious bovine pleuropneumonia, which is caused by a mycoplasma. For almost all these diseases during their chronic phase, the pathogenic agent has been reported to be present in semen. Semen transmission has been well established and documented for several such agents such as in particular Foot and Mouth Disease and blue tongue in ruminants. Almost 80 diseases are listed by the OIE in List B and nine of them can affect multiple species. There are other

important diseases not listed which must be also considered such as BVD for example. Of the viral diseases in List B, those that have been investigated the most are enzootic bovine leucosis (EBL), the herpes virus disease so-called, infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/PV). The EBL virus is exclusively associated with blood cell, so contamination of semen from infected animals is only possible if blood cells are included. IBR/IPV has been the subject of considerable investigation. As with most of herpes viruses, bovine herpes virus (BHV1), the pathogen of this disease, has phases of excretion and phases of latency. Intermittent excretion of BHV1 in semen has been shown to occur in bulls (Guérin, 1989) and reactivation of latent infection without warning or noticeable signs is a great hazard. The BVD virus has been identified in semen and the major risk arises from the so-called " persistently infected" animals which continuously shed the virus. Bulls with persistent infection, as well as those with the acute form of BVD, may shed the virus in semen and thus transmit the disease (Kirkland, et al., 1997). Numerous bacterial agents can also be associated with semen and among those a special care should be addressed to brucella and mycobacterium tuberculosis. With regard to cattle, it should be mentioned that prions do not pose a risk of transmission through semen contamination (Wrathall et al., 2000, 2001).

1.2. With regard to in vivo collected embryos, quite a number of pathogens have been investigated in their interaction not only with bovine but also with ovine/caprine and swine embryos, using different approaches: in vitro contamination of embryos, in vivo collected embryos from affected donors and search for the association of the pathogens to the embryo either in vitro or from recipients receiving possible infected embryos. Table 2 summarizes some of the data collated in the IETS Manual (1998). These results show that, provided that defined sanitary practices (see below) are followed during the process, the risks of transmission of a given disease from the donor to the recipient via an embryo is minimum.

1.3.As far as the in vitro produced embryos are concerned, the sanitary risks associated with the donor females have recently been reviewed by Guérin et al., (2000). The first point of interaction is the oocyte itself and its follicular environment, (surrounding cells of the oocytes and the follicular fluid). Contamination of such cells by two types of viruses, BVDV and IBR/IPVV (those two viruses are those that are the most commonly studied) has been reported by several investigators in different parts of the world (see the referred above review). Those viruses appeared to adhere to the oocyte zona pellucida and hence are "external" to the oocyte. It would be interesting to investigate further if a given pathogen could be found "inside" an oocyte collected from the ovary with then the possibility of interacting with the genome directly. Of course if that was the case, it could also theoretically occur in the natural process, except that here with the in vitro procedure, one collects oocytes that otherwise might never have spontaneously ovulated. To date and to our knowledge, no infectious agent has been retrieved inside the oocyte with the notable exception of a report by Bielanski (1994) on Camplylobacter fetus that suggested an intracellular contamination of that bacterium. Of course the conditions of this observation were totally experimental and it is hence difficult to assume that it mimics exactly the normal situation. This however should be more thoroughly investigated and could be a theoretical or potential challenge as no field evidence has arisen of infected progeny.

The zona pellucida of in vitro produced embryo seems to interact with pathogens differently than from in vivo embryos. One further evidence of this was reported by Marquant-LeGuienne et al., (1998) investigating quite an important pathogen in ruminants and swine, the FMD virus. These authors in vitro contaminated in vitro produced embryos and showed that the 10 washings of the embryo recommended were unable to remove the association of the FMD virus (type O) from the embryo as opposed to what was reported for in vivo derived embryos (Singh et al., 1986 and see review in the IETS Manual). It remains to demonstrate if the other types of FMD virus behave in a similar manner and if enzymes such as trypsin would be effective for each of the types of the virus in dissociating them from the in vitro produced embryo zona pellucida. Various bacteria have also been investigated and a recent report on another type of pathogen, Tritrichomonas foetus, has recently shown that this parasite experimentally associated with in vitro produced bovine embryos was not further detected in embryonic cells of ZP intact embryos or hatched embryos after culture hence rendering the potential risk of transmission unlikely (Bielanski et al., 2004).

1.4. The possible sequence of hazards. As proposed by Thibier and Guérin (2000) for the in vitro production of embryos, the sequence of hazards in terms of infectious agents includes (1) those related to the female donor and the mode of collection (abattoir collection or ovum pick up), (2) the maturation process, (3) the fertilization (introduction of semen), (4) the co-culture in vitro development, (5) the cryopreservation before (6) the last step, thawing and transfer. The sequence is similar for artificial insemination and for transfer of in vivo collected embryos with their relevant "routes".

For semen donors, the environment in which the animal is located has the utmost importance in terms of likelihood to be in contact with a given pathogen. The same holds true for embryo donors. For the latter, Stringfellow and Givens (2000) nicely summarized from an epidemiological point of view, the risks at stake. "If a pathogen was to be transmitted by transfer of in vivo derived bovine embryos, an uninterrupted sequence of events would have to occur". This sequence includes (1) the exposure to pathogen, (2) the continued association of pathogen with the embryos, (3) the maintenance of infectivity of pathogen throughout embryo manipulation and processing and finally (4) delivery of an infective dose of pathogen to a susceptible recipient. Several factors well identified in the review of Stringfellow and Givens (related to the own properties of the embryo such as those of the zona pellucida or to the handling and

procedures used: washing, antibiotics etc.) give some explanation to those observations.
This can be extended to all reproductive technologies and greatest care is always to be taken to ensure that there is no addition of pathogens or contaminants during the whole process.
A special notice should be given to the risks associated with materials of animal origin. Any biological product of this kind used for recovery of gametes, sperm and oocytes or embryos, dilution, in vitro maturation of oocytes, washing and storage is potentially a source of contamination. This is of particular relevance with regard to the Transmissible Spongiform Encephalopathies (TSE) as discussed at large by Wrathall, 2000.
The putative contamination of semen or embryos while stored in liquid nitrogen (LN) tanks for example, as an additional source of contamination, has received recent attention. Bielanski et al., (2003) have demonstrated the occurrence of microflora in LN tanks such as Stenotrophomonas maltophilia that was able in experimental contact with semen to decrease the motility of semen. These authors have indicated that direct contact of contaminated LN with embryos may lead to their association with viral agent. However, they have also shown that all sealed samples of embryos stored in contaminated LN tanks tested negative for the presence of bacteria or viruses.

For in vivo-collected embryos, and with the proviso that all guidelines published by IETS and OIE after official approval, are rigorously followed, the IETS relevant committee, namely the Health and Safety Advisory Committee (HASAC) has categorized diseases according to the risks assessment analysis, into four categories. The category one is "that for which sufficient data are available to determine the risks to be negligible provided that the embryos are properly handled between collection and transfer". As seen in the table 3, there are only seven diseases listed in this category and it is unlikely, unfortunately, that this number will increase in the near future due to the insufficiency of research in this area.

2. THE RISK MANAGEMENT.

If for the risk assessment section there are similarities and dissimilarities between semen and embryos, clearly for the risk management, the procedures to be followed and accordingly the official recommendations are radically distinct.

2.1. For semen, the basic epidemiological rule is the following: for a given pathogen, the semen may be guaranteed to be free of a given pathogen if the semen donor is free from it **and** if the donor is one of a group of individuals that are free from it. This approach requires a very strict and well-monitored system of control of the male studs. Specifically, the semen should be collected (i) in an approved semen collection center (SCC), (ii) in a hygienic manner by technically trained and experienced people and (iii) under a rigorous program controlling the health status of the sires. The quarantine station in which the bulls are to stay prior to their entry to the SCC is important. The station management should ensure that only individuals free of specific diseases enter the SCC. If this quarantine station is the primary line of defense, the second refers to the adequate design and management of the SCC. Its general organization should be officially approved by the veterinary authorities according to the recommended guidelines and the center should adhere to a biosecurity program under a quality assurance system. Guidelines are all based on recommendations laid down in the OIE Terrestrial Animal Health Code Appendices: 3.2.1, 3.2.2 and 3.2.3. (OIE Code, 2003). One of the prime examples of the application of these measures is the EU Council Directive on semen, referred to as "Directive 88/407 for the bovine species.
The processing laboratory must also be monitored; only authorized personnel should be allowed to enter. The basic organizing idea in such a laboratory is the FORWARD rule: once the semen has been collected, it should move forward from one place to another with no return or crossing. Health surveillance and testing is also to occur according to the recommendations provided in the OIE Code. The principles are first, to control and monitor individuals prior to their entry into the quarantine station and further prior to entry in the SCC. Health considerations of the area, herd of origin and each individual animal must be considered. Second, regulation examination and testing of the males in the center must be performed. Three major types of monitoring are required at regular intervals (once or twice a year): (i) thorough clinical examination of all individuals, (ii) detailed andrological examination of the collected semen and (iii) complete testing for various diseases recognized as the major sources of risk, such as tuberculosis, brucellosis, IBR/IPV, BVD, campylobacteriosis, tritrichomoniasis etc…

2.2. For embryos collected in vivo, the practical procedural guidelines for collecting and handling the in vivo derived embryos are described in details in the IETS Manual. It should be considered as a code of good practice and could be included in a quality assurance system wherever possible. The first step of course refers to the thorough clinical examination of the donor animal and its environment (lack of infectious contagious disease in the area or in the herd). For the handling of embryos, the basic recommendations are as follows. The first stage is to ensure an appropriate washing, 10 times consecutively with a new pipette each time, with immersion of the embryo(s) in each wash for duration of 1 min. with light agitation and with at least a dilution factor of 1/100 between each washing. There are now means to do this in a convenient manner and consuming little time. The embryo should be very carefully inspected under magnification (X 50) and should only been processed if the embryo has an intact zona pellucida and no adherent debris because such cells could serve as a source of contamination and allow for carry over the pathogen. The treatment of embryos with the enzyme trypsin is often recommended when dealing with "sticky" pathogens such as the herpes virus BHV1. This was shown not to be always necessary (Thibier and Nibart, 1987) but is nevertheless a good procedure and often required for exported embryos. The way trypsin is to be handled is also relevant since as a protein enzyme, it is

quite sensitive to the environment. It should also be mentioned that such a treatment is not by any mean, a panacea. Even if used, it should not be considered as replacing the need for sanitary precautions with the environment of the embryos.
The media may also be of some concern as discussed above. Its nature and origin should hence be selected with great care. The addition of antibiotics is also of some value if used appropriately. The quality control of the whole process is now necessary for a given team (see below) and regular testing in the media collected and stored for assay should be a standard procedure. This could involve search for a putative contamination by various viruses that might originate from the collected donor of from some serum used in the media, and the status for pathogenic and also for saprophytic microflora. This should contribute in the mid-term to establish and verify the effectiveness of the quality assured production process procedure.
These procedural considerations are part of the OIE recommendations (Terrestrial Animal Health Code: Appendix 3.3.1.) that specifically refer to the guidelines published in the IETS Manual. They are also most of the time included in the regulations for moving embryos from one farm to another. In doing so, it is right to state that embryo transfer contributes to improving the animal health status of a given population in controlling very strictly such movements of germplasm between herds. The basic concept of those regulations relies on that of the official approval of embryo transfer teams. This was a very important step in the scope of the veterinary regulations that generally rely on the animals, its confinement and its products. Here the safety of the industry fully relies on the ethical and technical excellence of the man/woman in charge, head of the embryo transfer team. The criteria conventionally used by the veterinary authorities to give their official approval relies on four major points:

(1) the supervision of the team by one veterinarian, with often the requirement for adequate training in terms of hygienic procedures for the personnel involved,

(2) the necessary equipment to proceed adequately to the different steps of the procedure,

(3) the commitment of the head of the team to strictly follow the procedural guidelines as stated in the IETS Manual,

and (4) be regularly submitted to official tests of flush fluids, washing fluids and degenerated embryos in terms of possible viral or bacterial contamination.

These teams are under the overall supervision of the official veterinary authority and are regularly inspected.

2.2. For the in vitro produced embryos, a set of recommendations to control risks associated with such embryos have been elaborated within the IETS and been published in the relevant chapter of the IETS Procedures Manual. Here too, they should be considered by all practitioners as a mandatory code of good practice. The first step to survey is the health status of the area, the herd of origin when relevant and the donor herself making sure that no infectious, contagious disease are present at the time of collecting the oocytes. A special note is to be given when dealing with animal from a given species or breeds threatened by extinction. It may well be for reasons of biodiversity or germplasm conservation that the general conditions required are not met. There could consequently be some exceptions, because of the considerable power of this technique for quality control (see below) and this, incidentally, constitutes one comparative advantage to this technique. When ovaries are collected from the slaughter house, it is of the greatest importance to trace back the herd situation of those females and check for example that they do not come from any depopulated herd for health reasons. The premises and working areas should be so designed that individual specialized units are set aside for particular tasks with restricted access. Wherever possible, a laminar flow chamber should be in place with close attention to cleaning and disinfecting procedures as rightly stated by Guérin et al., (2000).
The handling of embryos during the various steps should always be conducted with great care and under highest hygienic conditions. The quality of the media and of the co-culture cells system when relevant is one of the most critical point of the procedure. All biological products should be strictly controlled and guaranteed free from microorganisms (virus, bacteria or fungi). Sera containing antibodies against agents of particular concern should be avoided. It is also strongly advised to have knowledge and confirmation of the inactivation procedures from the manufacturers when relevant.
Adding antibiotics to the media is also always of good practice as it contributes to remove permanent or opportunistic pathogenic agents or saprophytic microorganisms inadvertently introduced at the collection point or at the time of fertilization from semen that can never be sterile (Guérin et al., 2000). Finally, the recommended washing procedure such as that described above for the in vivo derived embryos contributes to further reduce the likelihood of associating pathogens with the embryos so produced and released from the lab for transfer. The interest of adding trypsin is still a matter of debate as insufficient studies have been yet performed to assess the advantage of such a procedure with no detrimental effect. One of the major comparative advantages of this technique is that the production system provides control points and sufficient time to allow for each batch of embryo produced to be monitored and assessed to relative to their sanitary status. In addition, the many different media used provides an excellent source of sampling as it has been shown that the media, as a mediate environment of the embryos, serves as a good indicator of the pathogens to which they could have been exposed during the process (Thibier et Guérin, 1993). The quality control is here of particular relevance.
As for in vivo derived embryos, based on full consideration of relevant scientific peer reviewed papers, an Appendix (Appendix 3.3.2.) in the OIE International Animal Health Code for in vitro produced embryos has been elaborated and approved, with subsequent adoption of national regulatory frameworks such as the European Union Directive (89/556 modified 93/52 and 94/113).
The official guarantee of safety in terms of animal diseases relies on two factors: first only an officially approved team is allowed to process such embryos and

second such teams are under the control of the veterinary authorities of a given country (Thibier, 1993).
As for in vivo derived embryos there are four major conditions to be met for the teams to be officially approved:

(1) the supervision of the team by a well trained veterinarian in terms of hygienic and sanitary procedure,
(2) the capacity of the team to work in satisfactory conditions with particular attention to the premises, arrangement of the lab and equipment
(3) the commitment of the team to strictly follow the procedural guidelines as referred to in the IETS Manual,
(4) the regular submission of the team to the inspection of the veterinarian authorities and to sanitary controls of the degenerated or non- fertilized embryos, maturation and culture fluids stored for this purpose.

CONCLUSION

In conclusion, when moving gametes or embryos, there are definitely risks at stake of associating pathogens with them. However, as those Reproductive Biotechnologies developed, the veterinary community has devoted a considerable number of investigations both in vivo and in vitro to make clear assessments of those risks. This has led to recommendations and guidelines validated by the relevant intergovernmental Agency, as far as the control of infectious diseases are concerned, namely the Office International des Epizooties. There is still room for further research for some agents, particularly for exotic diseases and this should certainly be encouraged. However, the system in place worldwide has proven to be effective with considerable numbers of such transfers. It is based on science and integrity in the collection and processing procedures. It is hoped that when the new Reproductive Biotechnologies such as nuclear transfer or transgenesis when appropriate will be implemented, the same approach will be taken by both scientist and regulatory agencies.

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Table 1. List A diseases in mammals susceptible to be transmitted through AI (from Thibier and Guérin, 2000).

Disease or pathogenic agent	Bovine	Ovine/ Caprine	Porcine	Equine
Foot and Mouth Disease	P; Tr	P;Tr	P; (Tr)	
Vesicular stomatitis	(P); (T)			
Swine vesicular disease			P; Tr	
Rinderpest	P; (Tr)	P; (Tr)	(P)	
Peste des petits ruminants		P; (Tr)		
Contagious bovine pleuropneumonia		(P); (Tr)		
Lumpy skin disease				
Rift Valley Fever	P; (Tr)			
Blue Tongue	(P)			
Sheep pox and goat pox	P; Tr	P; T		
African Horse sickness		P; (Tr)r		
African swine fever				(P)
Classical swine fever			P; Tr	
			P; Tr	

P: presence demonstrated; Tr: transmission demonstrated; () highly probable

Table 2. Summarized results of studies of pathogens- intact zona pellucida embryos interaction (derived from the IETS Manual, 3rd ed., 1998)

Types of pathogens	No. of embryos exposed (*)	Assay of embryos
In vitro contamination and assay of bovine embryos (**)		
Viruses	12 - 169	0
Other viruses (***)	29 - 144	36 to 100 % positive
Bacteria	38 - 96	0 –26 % positive
Mycoplasmas	20 - 111	30 – 100 positive
Assay of embryos from zona pellucida intact bovine embryos from infected or seropositive donors		
Virus	2 – 372 (****)	Negative
Brucella	309	Negative
Chlamydia	5	Negative

(*) range of number of embryos per pathogen studied
(**) high concentration exposure mimicking a “worse case scenario”.
(***) BHV-1, BHV –4, VSV.
(****) FMD virus-infected donors.

Table 3. List of IETS/OIE diseases in category 1[1].

DISEASE	SPECIES	NOTE
Foot and Mouth Disease	Cattle	
Enzootic Bovine Leucosis	Cattle	
Bluetongue	Cattle	
Brucella abortus	Cattle	
Infectious Bovine Rhinotracheitis	Cattle	Trypsin treatment required
Pseudorabies	Swine	Trypsin treatment required
Bovine Spongiform Encephalopathies	Cattle	

[3] according to the conclusions of the Research sub-Committee of the IETS HASAC (OIE, Terrestrial Animal Health Code, Appendix 3.3.5,2003).

[1] Special categorization for in vivo derived embryo-pathogen interaction