Disease control: procedures for handling embryos*

E.L. SINGH**

Summary: In order to avoid the transmission of pathogens by embryo transfer, several criteria must be respected. Firstly, the embryos must be manipulated with sterile material in a vector-free room. After thorough examination of the zona pellucida, the embryos are gently agitated in ten successive washes. They are then processed by trypsinization and by five washes in phosphate buffered saline in order to eliminate pathogens.

The uterine flush fluid, washing fluids, and the degenerate or damaged embryos are used for control testing, which should be carried out within 24 hours. After freezing some viral inactivation can occur.

KEY-WORDS: Cow - Disease control - Embryo - Testing procedures - Transplantation - Washing.

In terms of disease control, the disadvantage of using embryos for the introduction of genetic material is that, at the present time, the embryo cannot be tested and transferred, as the testing procedure kills the embryo. Its health status must therefore be inferred.

This disadvantage, however, is more than balanced by the apparent disease control potential of early bovine embryos. Research has shown that zona pellucida-intact bovine embryos are remarkably resistant to infection (1). In order that disease agents not be transferred along with the embryo, however, it is essential that embryos be collected, washed and processed in such a way that no pathogenic organism be introduced. The following criteria have been developed as a general guide on the handling of embryos to ensure disease control.

BASIC REQUIREMENTS

A clean, vector-free room, away from the animals, is required for the searching, washing and freezing of the embryos.

All media, sera, solutions, equipment, containers and ampoules/straws used in the processing of embryos must be sterile.

Antibiotics should be included in both the flushing and washing fluids.

Only embryos from the same donor should be processed together.


** Agriculture Canada, Animal Diseases Research Institute, Nepean, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9, Canada.
A clean room is sufficient for the processing of embryos. It is not practical to use a biological containment hood since embryo processing requires the use of a microscope.

In order that embryos do not stick to the surfaces of containers, they require protein in the medium. The sterility of this protein component can pose a problem. The use of either bovine serum albumen (BSA) fraction V or gamma-irradiated serum might ensure sterility; however, additional work is needed before a final decision can be made. Certainly the process of manufacturing the BSA fraction V would have to be standardized and accepted. There is also some evidence to suggest that the temperature at which gamma irradiation takes place is an important factor in ensuring sterility. Until a universally acceptable product becomes available, the serum component used in the flushing and washing medium should be certified virus- and mycoplasma-free and must either originate in or be authorized by the importing country.

**INSPECTION OF EMBRYOS**

The zona pellucida of each embryo should be examined over its entire surface area at not less than 50x magnification and certified to be intact and free of adherent material.

**Comments/discussion**

To ensure that the zona pellucida is intact, the embryos should be gently rolled in the dish so that all surfaces of the embryos can be inspected.

**WASHING PROCEDURE**

Embryos from the same donor should be washed in groups of ten or less through ten changes of medium. The embryos must be gently agitated in each wash and the micropipette used to transfer the embryos must be changed after each of the transfers. Each wash must constitute a one-hundred-fold dilution of the previous wash.

The zona pellucida of the embryos must still be intact after the washing procedure has been carried out.

**Comments/discussion**

Embryo washing must precede embryo freezing. Freezing can result in damage to the zona pellucida and thus any pathogens present must be removed prior to carrying out freezing. Once the zona has been cracked and a virus has entered the embryonic cells, further washing would be of no avail.

The recommendation of ten washes with each wash a one-hundred-fold dilution of the previous one is very conservative. *In vitro* experimentation has shown that very high levels of pathogens ($10^7$ to $10^{10}$) can be removed from embryos with fewer than ten washes (Singh, unpublished data).
Only ten embryos from a donor should be washed together. Again, this is a very conservative figure, and is based on considerable in vitro experimentation (3). There is no justification for washing embryos individually. This is especially true if the importing country will only accept the embryos after the uterine flush fluid is tested and found to be negative. In this instance, it is "the embryos collected from a single donor" and not the "individual embryos" that constitute the single entity.

One of the most important requirements to ensure proper washing is the gentle agitation of the embryos in each of the washes. As long as this is done, the embryos do not have to sit in the washes for any length of time, they may be moved to the next wash by the time it takes to change the transfer micropipette.

Flat bottomed plates that have twelve 20 mm wells (Flow Laboratories, Inc., λ 76-053-05) have been proved useful in the washing procedure. Two ml aliquots of phosphate buffered saline containing antibiotics and fetal bovine serum are placed in ten wells. The embryos are then transferred through the ten washes using disposable 20 μl micropipettes (Fisher, λ 21-164-2D).

**TRYPsin TREATMENT**

Embryos are transferred through five washes of phosphate buffered saline containing antibiotics and albumen, then through two aliquots of 0.25% trypsin, pH 7.8-8.0, for a total time in the trypsin of one to one and a half minutes. After trypsinization, the embryos are transferred through five washes of phosphate buffered saline containing antibiotics and serum.

**Comments/discussion**

Since trypsin is inactivated by serum, albumen must be used in the five washes that are carried out prior to the trypsin treatment.

Trypsin does not remove the zona pellucida from embryos. In fact, there is no difference between untreated embryos and trypsin-treated embryos when they are examined microscopically.

Trypsin is very effective in rendering embryos previously exposed to \(10^7\) pfu/ml of infectious bovine rhinotracheitis virus non-infectious. However, the effect of the trypsin treatment on the pregnancy rate obtainable with these embryos has not been fully determined.

**PREPARATION OF SAMPLES FOR TESTING**

**Flush fluids:** The uterine flush fluid (collection fluid) should be placed in a sterile container, such as a measuring cylinder, and allowed to stand undisturbed for one hour. The supernatant fluid should then be removed and the bottom 100 ml, along with any accumulated debris, decanted into a sterile bottle, and retained. If a filter is used in the collection of embryos, then any debris that is retained on the filter must be rinsed into the 100 ml of retained fluid.

**Washes:** The last four washes of the embryos should be pooled and retained.

**Embryos and unfertilized ova:** All degenerating embryos and unfertilized ova collected from a donor should be pooled, washed ten times and retained. Prior to assay, the embryos should be disrupted by sonication.
Comments/discussion

Which of the above samples should be tested in lieu of serological samples from the donor and sire is a matter that each importing country must decide on. It should be emphasized, however, that the testing of the flush fluid, wash fluid or embryos/ova for specified diseases should not be required if the donor and sire have been deemed free from these diseases by serological or other required tests. Pathogenic agents will not be found in the flush fluids, wash fluids or embryos if they are not present in either the donor or sire. It would be very important, however, to test the flush fluid and/or wash fluid and/or embryos/ova if the donor were seropositive.

The justification for testing the uterine flush fluid is that it gives some indication of what the embryo(s) might have been exposed to during their stay in the reproductive tract of the donor (i.e. it is an indicator of the health status of the donor). It is important that a sizeable sample of the flush fluid be tested in order that the results be reliable. If this is not possible, then the untested portion should be centrifuged at 1,000 g for 30 minutes, the pellet resuspended and tested.

The rationale for testing the last few washes is that it provides some indication of what the recipient will be exposed to when the embryo is transferred. For example, research has shown that bovine leukemia virus can be found in the uterine flush fluid of seropositive animals.

However, in these cases, the virus is removed from the embryos by washing as evidenced by the transfer of over 500 embryos from BLV-positive donors to BLV-negative recipients without the transmission of the disease (2). Again, if the washes are to be used as an indicator of embryo health then it is essential that the entire wash sample be tested. Thus, if each wash is 2 ml and the last four are to be tested, then all of the pooled 8 ml should be assayed. The justification for testing the last four washes is that it provides a very safe margin to ensure that proper washing has been carried out.

Testing the degenerating embryos and unfertilized ova collected from a donor provides some indication as to what the embryos have been exposed to and also whether proper washing has been carried out. The assumption is made that the health status of these embryos/ova represents the health status of the transferrable embryos from the same donor. To date, experiments with bovine embryos and ova would tend to support this theory — infectious bovine rhinotracheitis virus, for example, sticks to the zona of both embryos and ova regardless of their developmental stage and/or physiological state.

Storage of Samples

All samples (flush fluid, washing fluid and embryos/ova) should be kept at 4°C until tested. To maximize the reliability of the results, the testing should be carried out immediately, or at least within 24 hours of collecting the samples.

Comment/discussion

If testing cannot be carried out within 24 hours then the samples should be stored at –70°C. It must be recognized, however, that freezing can result in some viral inactivation.

*   **

Résumé : Plusieurs critères doivent être respectés pour éviter la transmission d'agents pathogènes par les embryons. Il faut tout d'abord les manipuler avec du matériel stérile dans un local exempt de micro-organismes. Après un examen minutieux de la zone pellucide, les embryons sont agités doucement au cours de dix lavages successifs. Ils sont ensuite soumis à une trypsinisation et à cinq nouveaux lavages dans une solution tampon pour favoriser l'élimination des agents pathogènes.

Le liquide utérin de récolte des embryons, les liquides de lavage et les embryons dégénérés ou endommagés sont utilisés pour les examens de contrôle. Ces examens doivent avoir lieu dans les 24 heures qui suivent la manipulation des embryons car la congélation peut inactiver certains virus.


*  
**


Resumen : Se han de observar varios criterios para evitar que los embriones transmitan agentes patógenos. Primero, es preciso manipularlos con material estéril en un local exento de microorganismos. Tras un minucioso examen de la zona pelúcida, se agitan suavemente los embriones en diez lavados sucesivos. Seguidamente se los somete a una tripsinización y a cinco nuevos lavados en una solución tampón para favorecer la eliminación de los agentes patógenos.

Para los exámenes de control, se usan el líquido uterino de recolección de embriones, los líquidos de lavado y los embriones degenerados o estropeados. Se deberán hacer los exámenes en las 24 horas que siguen a la manipulación de embriones, ya que la congelación puede inactivar algunos virus.

PALABRAS CLAVE : Control sanitario - Embrión - Lavado - Métodos de análisis - Transplante - Vaca.

*  
**

REFERENCES


4. THOMAS F.C. & SAMAGH B.S. — The gamma ray sensitivity of some animal viruses and antigens at 4°C and −190°C. *Veterinary Microbiology* (submitted).