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BOVINE EMBRYO COLLECTION TECHNIQUES: 
WAYS TO IMPROVE EMBRYO RECOVERY RATES

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Weatherford, Texas, USA

Abstract

In the early 1970’s bovine embryo donor collections were performed by surgical technique. Tubal stage (8 to 16 cells) embryos were recovered. The number of corpora lutea could be counted relatively accurately with the naked eye and later compared to total ova/embryos recovered (Hasler, personal communication). In the mid 1970’s non-surgical methods were reported as being efficacious, and this led to a large increase in both beef and dairy commercial embryo transfer (ET) (Elsden, Hasler et al. 1976; Rowe, Del Campo et al. 1976). Today there are numerous methods of non-surgical embryo recovery techniques performed by individual ET practitioners. Variations are due to factors such as Foley catheter size, location of placement of the Foley in the uterus, volume of collection medium placed into the uterus during each flush cycle (fluid in plus fluid out), number of flush attempts, total volume of saline used for the entire collection procedure, duration of collection procedure, and type of equipment external to the Foley catheter. Collection medium can be introduced into the uterus via gravity flow or by the aid of a mechanical device such as a syringe. Also, uterine massaging techniques used to recover the collection medium from the uterus differ amongst ET practitioners.

Introduction

There are many factors including embryo recovery techniques that ultimately determine the number of total ova (viable embryos plus unfertilized and degenerate ova) collected from superovulated bovine donor females. Breed, parity, body condition, superovulation protocol, and management schemes, are just a few of the variables that can lead to differences in total ova ovulated and transported from the oviduct to the uterus where they are available for recovery (Stroud and Hasler 2006). However, once donor females have been superovulated, inseminated, and are ready for collection seven days post onset of estrus there are many different embryo collection techniques employed by ET practitioners. The purpose of this study is to discuss and evaluate ova recovery techniques independent of cow and management factors.

The percentage of ova collected relative to the number of corpora lutea (CLs) on both ovaries is the best indicator of embryo recovery technique, but most practitioners don’t record these data and the American Embryo Transfer Association (AETA) stats committee does not ask for it. Also, rectal palpation can often be misleading when counting CLs (Stroud, BK 1994). Transrectal ultrasonography is a more accurate tool to diagnose and count CLs, but ultrasound also has limitations especially in heavily stimulated donors. As a result of the lack of these pertinent CL data, recovery efficiency as defined by total ova recovered/total number of CLs will not be discussed. Instead, the details of embryo collection techniques from several practitioners who reported a relatively high number of total ova/embryos per donor collection will be discussed so that others may discover ways to improve their collection technique.

Few controlled studies have been conducted comparing embryo collection techniques but some have reported differences (Goncalves, Gregory et al. 1987, Sartori, et al. 2003). However, the AETA Statistical Information committee’s figures indicate a notable numerical difference in total ova per collection when comparing clinical data reported by member practitioners.
Table 1. AETA total ova recovered per stimulated collection 2007 dairy data (minimum 200 collections) (Stroud, B 2008)

<table>
<thead>
<tr>
<th>ETB</th>
<th># stimulated collections</th>
<th>Mean # ova recovered/collection</th>
<th>Difference from mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>328</td>
<td>16.6</td>
<td>+ 6.2</td>
</tr>
<tr>
<td>Low</td>
<td>299</td>
<td>5.8</td>
<td>- 4.6</td>
</tr>
<tr>
<td>US Industry combined</td>
<td>19781</td>
<td>10.4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. AETA total ova recovered per stimulated collection 2007 beef data (minimum 200 collections) (Stroud, B 2008)

<table>
<thead>
<tr>
<th>ETB</th>
<th># stimulated collections</th>
<th>Mean # ova recovered/collection</th>
<th>Difference from mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>620</td>
<td>16.9</td>
<td>+ 6.3</td>
</tr>
<tr>
<td>Low</td>
<td>438</td>
<td>9.0</td>
<td>- 3.6</td>
</tr>
<tr>
<td>Industry combined</td>
<td>34299</td>
<td>12.6</td>
<td>0</td>
</tr>
</tbody>
</table>

Beef and dairy data were separated and only data from practitioners reporting at least 200 stimulated collections were considered. Table 1 (dairy data) compares the high and low total ova recovered per collection. The industry mean of 10.4 is displayed as a reference. The difference between the high practitioner and low practitioner recovery is almost 11 total ova per collection. The high practitioner was 6.2 total ova above the industry standard. Although the beef data in table two was not quite as drastic the high practitioner collected 7.9 more ova per collection than the low practitioner.

Perhaps a thorough evaluation of embryo recovery techniques, especially from those reporting the highest numbers, could provide some insight to the rest of the industry, especially for those on the lower level of the spectrum.

It should be pointed out that superovulation protocols and FSH doses, along with management, certainly play an important role in the outcome of embryo recoveries, but such information is not considered in this study.

**Early history of embryo recovery techniques**

Early reports of embryo collections were performed by surgery three to four days post onset of estrus (Elsden, 1977). Donor females were put in dorsal recumbency under general anesthesia. A 15 cm ventral midline incision was made just anterior to the mammary gland and the uterus and oviducts were exposed. One oviduct was canulated with a glass catheter. The anterior uterine horn ipsilateral to that oviduct was injected with a small amount of collection medium via a blunt needle and syringe. Digital pressure was applied posterior to the needle with the thumb and forefinger so that medium would be trapped between the anterior tip of the horn and the uterotubal junction (UTJ). Firm pressure was applied using the thumb and forefinger to push collection medium anteriorly through the UTJ and into the oviduct to back flush the 8 to 16 cell stage embryos into the glass cannula. This technique was relatively effective. Seidel, et al. (1977) reported from 3116 ovulations, 1834 total ova collected were collected for a 59% recovery rate, but surgery was expensive and proved to be deleterious to some donors as it created oviductal adhesions that sometimes led to post surgical infertility.

In the mid 1970s there were reports of successful non-surgical embryo collections. Elsden et al. (1976) reported a mean of 6.9 total ova from 26 superovulated donors. The technique utilized a Foley catheter inserted into one horn and filling that horn via gravity flow to the size of a 42 day pregnancy in young cows and the equivalent of a 60 day pregnancy in older cows. The process of filling and draining was
repeated multiple times. Rowe et al. (1976) reported similar results from 15 superovulated heifers. He collected each uterine horn separately using 30 to 150 ml each flush (gravity flow method) for a total of five flushes for each horn. His ova recovery index was 54% based on the number of ova recovered per CLs counted. This was very similar to the recovery index for surgical flushes (58%) done by a different group during the same time period. When he increased the number of flushes per horn to eight his recovery index increased to 69%. Both of these investigators reported a 95% recovery rate of the fluid introduced into the uterus and recovered during each flush cycle (fluid in/fluid out). Since these reports in 1976 ET practitioners have modified many aspects of the non-surgical procedure and continue to do so today.

**Embryo recovery techniques**

As previously mentioned there are differences in technique involved in the non-surgical embryo recovery process. One of the variables is whether the recovery apparatus creates a closed or open system. A closed collection system is where inflow and outflow (drainage) tubing remains connected to the Foley catheter, usually via a Y junction, throughout the collection process, which protects the fluid from possible bacterial contamination. Usually, fluid flows into the uterus by gravity flow in a closed system, but some practitioners use manual pressure applied to the collection medium plastic bag to force fluid into the uterus. An open system utilizes a catheter tipped syringe attached to the Foley during inflow and outflow, but is detached from the Foley after each uterine flush for the purpose of depositing the collectus into a filter or a searching dish. While the syringe is uncoupled from the Foley the collection system is considered open.

Most practitioners place an embryo filter on the end of the drainage tubing of a closed system. The 70 micron mesh is suitable to trap the ova (about 150 micron diameter) and it allows other small endometrial cells and collection medium to pass through the filter. The filter is rinsed post collection into a gridded plastic search dish.

There are advantages and disadvantages to both the open and closed system. The advantage of the open system using a syringe is that the volume of inflow can be measured precisely. The technician can then massage the uterus and occasionally put slight negative pressure on the syringe to facilitate recovering nearly all of the fluid put into the uterus. Technically, an input syringe can be utilized in a closed system if placed between the source bag and the Foley by utilizing a 3-way stop cock. The inflow could be measured accurately, but the outflow, post filter, would have to incorporate a measuring beaker of some sort as a reference to measure outflow. However, a closed system using a syringe would lose the advantage of the negative pressure allowed by the syringe in the open system during fluid recovery.

Another variable in the embryo collections process is placement of the Foley catheter in the uterus. Some technicians place the Foley into a single uterine horn and therefore perform unilateral horn recoveries. Practitioners using the single horn technique differ on where they place the tip of the Foley. Some describe a more anterior placement towards the tip of the uterine horn whereas others inflate the Foley balloon more caudally in the horn at a point somewhere near the external cornual bifurcation (Sartori, 2003). Still others place the Foley near the greater curvature of each uterine horn. Unilateral recoveries have the advantage of being diagnostic with regards to viable or non-viable ova being produced from either the left or right side of the reproductive tract. Also, the patency of each oviduct can be tested with this technique by comparing the number of CLs to the number of recovered ova from each horn. Some technicians use the unilateral horn flush, but collect both horns in the same drainage filter. The advantage of diagnostics is lost, but this saves time in the lab having only to rinse one filter and searching through only one search dish for ova. Many practitioners perform what is called a “body flush”. They place the Foley catheter in the uterine body with the balloon being seated caudally up against the internal cervical os. Both horns can be filled simultaneously or sequentially with collection medium, and drained individually or collectively. The advantage of the bilateral horn technique is that the chance for bacterial or fecal contamination is minimized since the system remains closed throughout the collection process. A unilateral horn flush requires unhooking the Foley from the inflow source and replacing a stylette into the
Foley and manipulating it back into the other horn for flushing. This creates an open system albeit for a short period of time. Supposedly, one of the advantages of a unilateral horn flush is that a smaller overall volume of collection medium is needed to flush the anterior portion of each horn, which is where the majority of the embryos are located.

The volume of collection medium placed into the uterus during each flush cycle seems to differ from one practitioner to the other, especially those who use the body flush technique. Some technicians initially place a small volume of 10 to 20 ml into the uterus. Once that has been recovered, based on palpation or volumetric measurement, each sequential flush cycle tends to be larger in volume, i.e., 20 to 30 ml for the second flush cycle, and 40 to 50 ml for the next until a maximum inflow of about 150 ml (approximately 75 ml into each horn). Other embryo collection technicians report an initial inflow of 150 ml or more from the onset of the process. No matter what the volume of inflow, most practitioners agree that gentle, and sometimes aggressive manipulation of the uterus is required to help initiate and complete the outflow of the collection fluids.

Another variable in embryo collection techniques is the number of flush cycles per collection. At least one practitioner that uses a 60 cc catheter tipped syringe for inflow and outflow (open system) reports utilizing only three to four flush cycles per donor collected. Some technicians using a body flush claim to use from 10 to 15 flush cycles per donor while others report about half a dozen cycles. There are several factors that determine how many flush cycles that would typically be used on a donor; 1) ease of outflow, 2) size of uterus, 3) stimulation or number of CLs present, 4) number of donors being collected or work load, 5) amount of mucus being produced by the collection procedure, and 6) experience of technician.

The total volume of medium used for the entire collection procedure varies among practitioners. Some elect to use a pre-determined amount, i.e., 150 ml per donor, whereas others use a liter or more. Still others do not use a set amount. Instead, they flush until, in their professional opinion, they have done a thorough recovery.

The duration of a collection procedure is not universally agreed upon. Very few technicians set a timer and quit when it goes off. However, some technicians collect as many as 20 or more donors in a work day, which requires either a short collection duration or not much sleep. Others report as average of 30 to 45 minutes, but most agree that the duration of a donor collection depends mainly on how fast the in and out flow proceed. A collection that produces a lot of sticky mucus can drain very slowly and often clogs up a filter, which adds considerable time to the collection process and embryo searching. The author has had a few collection procedures take up to two hours.

**Detailed description of an open system collection procedure**

One ET practitioner (A. Mills III, Zachary, LA) described the following open body collection technique. He passes an appropriate sized Foley catheter through the cervix and aims the tip into the left uterine horn. He inflates the Foley balloon with about two to three ml of flush medium (not air) to get a feel for its location. He then adjusts the location of the balloon so that it fits snugly against the internal os of the cervix. The size of the balloon is then adjusted by inflating it with an appropriate amount of medium so that he does not pull it back into the cervix during uterine manipulation and out flow. He also does not inflate the balloon too tightly for fear the inflow will be cut off to the opposite horn. When the Foley is seated appropriately he pinches off the right uterine horn with his thumb and forefinger and using a catheter tipped syringe filled with an exact volume of collection medium (25 ml for virgin heifers, 30 ml for first calf heifers, 35 ml for mature cows) he injects half the measured fluid into the left uterine horn. Once that is completed he pinches off the left uterine horn and injects the other half of the fluid into the right horn. Now that both horns are filled with collection medium he strips or massages each horn from the body anteriorly to make sure that fluid makes its way to the anterior tip of each horn. He then massages each horn for a few seconds to dislodge ova away from any possible endometrial folds and begins his drainage procedure. He puts very slight negative pressure on the syringe, but mainly depends upon positive pressure from the massage process on the horns to recover every ml that he placed in the
tract. The second flush cycle is similar to the first with the exception that he places all of the original (25, 30, or 35 ml) volume into the left uterine horn only. The same stripping and massaging process is used to force fluid to the tip of the horn and then the recovery process is initiated. Even though the input of the second flush cycle is aimed to the left horn only, it is important to massage both horns during the outflow because some of the fluid will rush past the tip of the Foley and into the right horn. So, the right horn will have to be massaged and evacuated to recover 100 percent of the inflow fluid. The third flush cycle is aimed at the right horn only. It is a repeat of the second flush cycle, but all inflow is directed into the right horn. The outflow process is identical to the second flush. After the third flush cycle has been completed the embryo search technician reports to the collection technician how many total ova have been recovered, and if the number of ova recovered corresponds to the number of CLs palpated he will quit. Otherwise, he will do a 4th flush cycle with fluid placed in both horns. Most of the time the 4th flush cycle is not necessary, but on occasions he may have to do more than four flushes. His total estimated time from start to finish is 30 to 60 minutes. There is a cost advantage to using this technique. Usually, less than 150 ml is used for each donor collected. Also, a filter is not necessary with such a low volume of flush fluids. Over the last two years, this ET practitioner has averaged 19.7 total ova per collection (64 total collections). He has been performing non-surgical embryo collections since 1977.

Detailed description of a closed system collection procedure

Another practitioner (B. Stroud, Weatherford, TX) described his closed body collection technique. The donor female is palpated and an appropriately sized Foley catheter is selected. Usually, the largest sized Foley that will easily pass through the donor’s cervix is chosen. Once the Foley has been passed through the cervix and into the uterine body the balloon is inflated exactly like the previously described method with the exception that the Foley tip is aimed towards the horn corresponding to the ovary with the most CLs. Using a gravity inflow system, about 20 ml of flush medium is placed into the uterus. No attempt is made to push the fluid toward the anterior tip of the horn. The outflow clamp is opened and an attempt is made to completely recover 95% or more of the inflow flush medium. The first flush cycle is usually “dirty” with cervical mucus and cells that passed into the Foley while passing through the cervix. If the mucus is severe, the outflow will not be allowed to go into the filter. Instead the filter will be detached from the outflow tubing and the mucus will be dumped into a sterile gridded search dish. Allowing heavy cervical mucus to contact the filter will create a great deal of difficulty when trying to rinse it. Embryos can be lost during the process. Once a clean outflow has been established the rest of the collection fluids can be passed through the filter. The second inflow volume will be approximately 25 to 30 ml with about half being diverted to each horn. The goal of the recovery technician is to remove greater than 95% of the flush medium from both uterine horns during each flush cycle. With each passing flush cycle a larger volume of flush medium will be allowed to inflow into the uterine horns. However, seldom will more than 75 ml per horn be placed into the uterus. Normally, about 7 to 10 complete flush cycles will be performed on each donor. The number of flush cycles is largely determined by how easily the outflow came from the cow, and how completely the fluid was evacuated during each cycle. Once the practitioner is confident that he has done a thorough recovery he quits, but leaves the Foley in place until the collectus has been searched. If after searching the number of ova does not closely compare to the number of CLs he will resume the collection process for at least two to three more flush cycles. A resumption of the collection process is considered a re-flush. Approximately 20% of his donors get re-flushed. About 50% (98/188) of the re-flushes in the last 12 months yielded at least one additional ova. (Neto, et al., 1985) reported that double flushing increased the average recovered total ova (P < 0.05) from 8.3 +/- 0.4 to 12.7 +/- 0.7 in Limousin and from 7.9 to 11.5 in Guzera. Also, utilization of double flushing increased (P < 0.05) the number of viable embryos from 4.7 +/- 0.3 to 6.9 +/- 0.5 in Limousin and from 4.5 +/- 0.4 to 6.4 +/- 0.7 in Guzera. His double flush was defined as follows: the first flush was completed when one liter of flush medium was put through the female’s uterus using a traditional closed system. The second flush consisted of filling the uterus of the donor with 80 to 150 ml of flush medium and letting her out of the chute to walk around for 30 minutes with the Foley catheter still intact. She was restrained again at that time and the flush medium was drained and searched.
Additionally, the author always drains the Foley catheter into a separate searching dish when the collection process is considered complete. Care must be taken to clean the vulva and external vagina free of fecal matter before extracting the catheter or gross contamination will result. With both the inflow and outflow tubing clamped off the Foley balloon is deflated completely. The Foley is gently extracted from the cervix and then drained into a separate searching dish. A small amount of fluid (approximately 3.5ml/16 French, 5 ml/18 French, and 7ml/20 French) will drain from the Foley catheter. Any viable embryos recovered from the Foley will be placed in a separate holding dish and washed independently of the embryos from the main collection procedure. That is done to prevent possible fecal contamination encountered when pulling the Foley from the cervix. In the past 12 months this practitioner has recovered 136 viable embryos from drained Foleys (923 donor collections). The record number of viable embryos recovered from a Foley is eight. Two other times seven viable embryos were recovered (prior years) from drained Foley catheters. This technician’s average total volume of fluid per donor collection is 400 to 700 ml. The average time to collect a donor is approximately 25 to 40 minutes. About 1 of 10 collections will take 50 minutes or longer. This practitioner averaged 15.6 total ova per collection (923 collections) over the last 12 months.

**Embryo searching**

Successful embryo collection procedures are important, but rinsing the filter into the search dish and finding the ova is essential. Many ET practitioners rinse filters with a medium that is free of surfactant. This prevents foam from covering the fluids during searching. The author uses a sterile 60 cc syringe with a one inch 16 gauge needle attached to rinse the filter. Using gentle flow the filter is rinsed with slightly over 50 ml into a sterile plastic square gridded dish and allowed to sit for two to three minutes so giving the ova ample time to gravitate to the bottom of the search dish. Post rinse the filter is visually examined to make sure the mesh is free of mucus. If mucus is still attached to the mesh it is rinsed until it is clean. Using a stereomicroscope the grids of the dish are systematically searched until all ova are found and transferred into a smaller holding dish. Once a dish has been searched completely the contents are stirred aggressively, especially the edges, with a micropipettor, and allowed to settle for a few minutes. The dish is searched a second time and aggressively stirred again and allowed to settle. Each donor dish is searched at least three times. If an embryo is found on the third search the dish is searched again, and the process is repeated until none are found. It is more common than not to find ova in the second search of a dish. About 15 per cent of the time ova are found in the third search.

**Unsuccessful recovery attempts**

Theoretically, for every CL on each ovary there should be a corresponding number of total ova recovered from each donor. During ovulation each ovum should travel directly from the follicle to the ciliated fimbria where they are swept into the ampulla of the oviduct. From there they make their way through the ampulla, past the ampullary isthmus junction (IAJ) then into the isthmus until about day four or five after the onset of estrus where they travel through the UTJ and dump into the tips of the uterine horns. For every CL that is palpated or scanned by real time ultrasonography an equal number of ova should be recovered. Unfortunately, it does not always work that way. Sometimes even repeated attempts at embryo recovery fail to produce the predicted number of total ova.

There are several possibilities for embryo collections with fewer than expected ova/embryos. The first deals with accurate CL counts. Rectal palpation for diagnosis of a CL, even for single ovulating cows, is only about 80 percent effective (Stroud, 1994). CL counts on heavily stimulated donor females can be inaccurate by palpation since the papilla of some of the smaller CLs can be sandwiched between two larger ones making them nearly impossible to feel. Also, some structures that are diagnosed by palpation as CLs are often later confirmed by ultrasound as unovulated follicles. That changes the expectations of the collection technician. In several cases, the author has predicted a dozen or so embryos based upon a rectal palpation CL count of 12 CLs only to recover zero total ova after a collection procedure. Further ultrasound scanning of the ovaries revealed that the palpated CLs were actually unovulated follicles. Even with ultrasound, depending upon the resolution of the unit, a perfectly accurate CL count can be difficult.
However, donors with a small to moderate number of CLs (1 to 8 per ovary) can be accurately counted (± 10%) by experienced ultrasonographers using high resolution equipment. To complicate matters, sometimeswhat appear to be unovulated follicles by ultrasound are instead unluteinized follicles that have ovulated but failed to produce any discernable luteal tissue on the periphery of the structures. Even with modern ultrasound units these unluteinized follicles can only be diagnosed retrospective of a collection procedure by counting only a few well defined CLs pre-collection, yet recovering numerous total ova and sometimes a high number of perfectly healthy embryos. In rare cases, a pre-collection ultrasound exam will reveal 100% apparently unovulated follicles only to find a dozen or more viable embryos after the collection procedure. Apparently, these follicles ovulate their ovum with only a minimal amount of follicular fluid escaping from the follicle yet enough progesterone is produced to maintain normal embryonic development. So, it is important to mention this ultrasound information, but assuming the CL count is accurate the difference between the number of CLs counted and the number of total ova recovered equals the number of unrecovered ova.

Unrecovered ova can be classified into three general categories as to root cause; physiological, recovery technique, and post recovery management of the collectus.

Physiological causes are due to ovum transport failure from the time of ovulation until the ova are transported into the uterus. Timing is sometimes the issue. Most ova are dumped into the uterus by day 5 after the onset of estrus. However, some will be discovered at day six, and yet some at day seven or eight. It is normal for a few ova to remain in the oviduct until day 9 or 10 post estrus. On at least two occasions the author has collected a donor with less than expected results, and then re-flushed the donor immediately with no results only to re-collect the cow the next day and recover additional viable embryos. Delayed ova transport to the uterus could also explain why US ET practitioners report only recovering ova only 60% of the time during a non-stimulated “single egg” collection procedure. Pathology can also play a role in transport failure. Adhesions involving the fimbria and infundibulum could prevent ovum uptake. Conditions like hydrosalpinx and salpingitis with swelling could also prevent ova transport. Tumors or metastatic cancer could also invade the oviduct and cause occlusion. Total blockage of both oviducts in the bovine is very rare, at least in the author’s experience.

Poor recovery technique is the most likely cause of unrecovered ova. As previously mentioned, the author re-flushes 20% of all donors collected due to a discrepancy between what was predicted and the number of ova collected after the initial collection procedure. Slightly over 50% of the re-flushes produce at least one additional total ova. About half of the ova recovered from re-flushes are viable embryos. This corresponds with the AETA stats on percent of recovered total ova that are viable embryos collected from stimulated beef donors (56% 2007).

There are some specific problems that could lead to poor embryo recovery.

1. Under inflating the balloon on the Foley could allow the tip of the Foley to be pulled too far posteriorly into the cervix, which could partially occlude outflow during flushing cycles. Sometimes the Foley bubble can be pulled completely out of the cervix.
2. Over inflating the Foley balloon can impede outflow of collection medium. It can also cause endometrial bleeding, which makes embryo searching more difficult. Anytime that outflow is compromised it can influence the percent of total medium recovered during each flush cycle.
3. It is possible that seating the Foley too deep into the uterus during a unilateral horn collection procedure could physically bypass any ova that have migrated from the tip of the horn to the greater curvature. A pre-collection ultrasound exam of the uterus will occasionally reveal 15 ml or so of intraluminal fluid in each horn. This fluid is not necessarily pathologic and it acts as a mechanical transport vector to move ova from the anterior tip of the horn posteriorly.
4. Failure to straighten out any kinks in the anterior uterine horns, both during inflow and outflow, could cause some of the ova to be trapped during flush cycles. The uterine horns of a bovine
female are rather long and tortuous. Once the uterus is filled during the inflow phase of a flush cycle that does not mean that collection medium has reached the anterior tip of the horn. Gentle manipulation and straightening of the tip of each uterine horn is necessary to insure that medium reaches the ova otherwise the collection technician may not access the ova at all. The same principle holds true for the outflow phase of a flush cycle. Collection medium may have accessed the ova, but any kinks in the long uterine horns will cause the ova to be trapped in a pool of medium and not recoverable. Manipulation of the uterine horns during outflow is a continuous process.

5. Can overfilling the uterus during an inflow phase of a flush cycle push ova back into the oviduct where they would be inaccessible for recovery? Close inspection of the literature will reveal that surgical collections (previously described) were accomplished by pooling collection medium into the anterior horn and forcing it to backflow past the UTJ and into the oviduct with pressure from the thumb and forefinger. Can ova be forced back through the UTJ during non-surgical flushing? It is highly probably that could happen is some cows. Early pioneers in non-surgical collections reported that prior to inflow they would pinch off the anterior tips of the horns for fear of back flushing the ova into the oviduct.

6. Excessive manipulation of the Foley balloon during the outflow phase of a flush can create heavy cervical mucus near the Foley outflow holes. This can block the speed of inflow and outflow, and sometimes completely stop flow. If the Foley bubble has seated too deeply against the internal cervical os the collection technician can push the bubble forward with thumb and forefinger placed on the posterior side of the bubble. This technique can improve outflow efficiency, but creates sticky cervical mucus which can stop up the filter.

7. The single biggest mistake made by collection technicians is failing to recover at least 95% of the inflow volume of collection medium from each flush cycle. In the early 1980s the author’s collection technique was an open system utilizing a 60 cc catheter tipped syringe for inflow, and a sterile glass Pyrex dish to catch the outflow. A technician searched each dish and reported to the collection technician as ova were found. The last few ml of each flush cycle contained most of the uterine cells and ova. Failure to be patient and to thoroughly manipulate the tract so that nearly all of the inflow is recovered from each flush cycle can lead to unrecovered ova.

8. Failure to drain the Foley can lead to collection failure. The Foley catheter represents dead space in a collection setup. If Y tubing is used for inflow and outflow, not only does the length of the Foley represent dead space, but so does the tubing from the Foley to the Y junction. Essentially, any fluid remaining in these dead spaces at the end of each outflow cycle would not enter either a syringe (open recovery system) or outflow tubing of a closed system. Ova can be caught in these dead spaces for up to 8 or 10 flush cycles. Draining the Foley into a separate search dish when the collection process is over can be profitable. This is especially so in donors with small reproductive tracts, i.e., virgin heifers or young cows. Smaller tracts allow for less inflow volume of collection medium. Therefore, the dead space per uterine lumen ratio is higher in cows with smaller tracts.

9. The number of flush cycles during a collection procedure can influence the number of ova recovered. Generally, five or six flush cycles per horn is adequate as long as the total volume of outflow is equal to the inflow (≥95%) per cycle. However, some collections require more flush cycles to recover most of the ova.

10. Filter management also plays a big role in ova collection efficiency. Filters should be managed so that mucus produced from the collection procedure does not impede flow. When outflow through a filter begins to slow it usually means the filter is clogged with mucus. It should be rinsed
immediately into a searching dish and replaced with a new one. It can take up to a half dozen or so rinses to completely clean a filter coated with cervical mucus. This makes searching for ova a very tedious and time consuming process. Ova can be lost in the process. During the collection procedure the recovery technician should monitor the filter for mucus and replace it at the first sign of a problem.

11. Embryo searching is an art form that is vital to finding all the ova that are recovered during the collection process. Inexperienced search technicians often overlook two classes of ova that could be the difference between a complete recovery session and one short of the total ova predicted; 1) embryos without a zona, and 2) ova embedded in a “cumulus nest” (see Figure 1). Usually ovulated ova lose their associated cumulus cells within a few hours post ovulation, but in some cases one or more ova become encased in a cumulus matrix. If not careful, these ova can be easily overlooked. These ova are just as likely to be viable embryos as not. They are easily teased out of the matrix by manual manipulation using two 20 gauge needles. Conception rates of cumulus matrix derived embryos are no different than normal embryos.

Figure 1. Four embryos embedded in a cumulus matrix or “nest) can be overlooked by inexperienced search technicians. Notice the morula and blastocysts outside the matrix.
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Keys to a successful embryo collection procedure

1. The collection technician must perform an accurate CL count, preferably by ultrasound, on each ovary prior to the embryo collection process.
2. Each flush cycle should result in greater than 95% fluid recovery from the uterus.
3. Avoid letting mucus onto the flush filter.
4. Avoid bloody flushes when possible. Do not over inflate the Foley balloon. Avoid being too aggressive when massaging for outflow. Be patient and gentle when having trouble with a stubborn recovery.
5. In a closed system make sure the outflow tubing is drained completely into the filter before detaching the tubing.
6. Always drain the Foley catheter after a donor collection
7. Compare the CL count with total ova collected and re-flush if necessary.

Conclusion

There are about as many methods utilized to recover embryos from superovulated donors as there are ET practitioners. Although this brief review of the embryo collection process only covers a couple of specific collection methods it does discuss some of the basic principles that all ET practitioners use in their day-to-day practice. In 2007 the AETA stats committee reported 54,080 (combined beef and dairy) stimulated donor collection procedures resulting in a total of 332,676 viable embryos recovered. The mean number of viable embryos per collection was 6.15. Any increase in the number of embryos collected by individual practitioners could have a positive financial impact to both the practitioner and the cow owner. Hopefully, this review of collection techniques will help some of the practitioners reporting on the lower end of the spectrum improve their recovery rates.

References

RECOVERING THE MISSING EMBRYOS

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As the title of my conference « Recovering the missing eggs » indicates, today we will look into cows that produce a number of embryos well below the number of corpus luteum observed. After a brief anatomical review of the bovine reproductive system, we will see the different steps our clinic ETE inc. has gone through, in order to set up a procedure and to better understand how to recover those famous missing embryos.

The bovine uterus, as with most hoofed animals and carnivores, is bicornual. It is made up of two ovaries, each connected to a uterine horn by a small winding canal called an oviduct at the end of which fertilization takes place. The two uterine horns meet to form a short uterine body which is followed by a cervix, the vagina, and the vulva. The oviduct is a very small tube which forms a continuation of the uterine horn. The oocyte penetrates in the oviduct by the infundibulum and progresses to a bulge called the ampulla, where the sperm, which have previously undergone their capacitation, have already arrived. Fertilization then takes place and the embryo makes its way toward the uterus, which takes 4-5 days after ovulation. There is no sphincter where the uterus and the oviduct meet. However, certain publications report folds of mucus aiming to hold back the sperm or even a physiological valve created by a vascular plexus in the lamina propria/submucosa of the junction wall. The possible existence of a structure where the uterus and oviduct meet will be important in our hypotheses explaining the results we came up with.

Since the beginning of embryo transfer, people have been convinced that embryos have practically no physical interaction with the uterus and were quickly withdrawn from the donor while collecting embryos. We would therefore consider that almost all embryos were removed after the first sprays of embryo collection liquid were given off and that, for safety reasons, there was good reason for more liquid to pass, so we took for granted that all embryos were removed from the donor. However, a certain number of donors presented an ovarian response well above the number of embryos collected, often with no explanation. Moreover, we noticed certain cases of donors which remained pregnant after having been collected, which indicated the presence of embryos in the uterus despite the embryo collection previously carried out.

During the 1997 CETA/ACTE convention, we had several discussions about “donors not giving all their embryos” with people from Embryobec, and they informed us of their procedure in such cases and the results they obtained. We then decided to attempt a second embryo collection in some selected cases and try to see what kind of results we would obtain. The first of the embryo collections gave us an oocyte the following day. We then realized that it was possible that some embryos-oocytes had remained in the uterus. The second safety embryo collection gave us two good embryos. The decision was then made to systematically carry out safety embryo collections the day following the first collection on those cows. Unfortunately, at that time, we did not document those cases very well, which prevents me from giving you more precise results. However, we then established a list of conditions that we judged necessary to justify a second embryo collection:

- At least one good embryo
- Ovarian ultrasound and the most precise possible counting of corpus luteum
- Good quality embryo collection liquid (lack of bleeding desired)
- A considerable difference between the ovarian response and the number of embryos collected the first time (Ideally a ratio superior to 2CL/emb)

At the beginning, the waiting period between the two embryo collections was one day. After that, due to our overloaded schedule the next day, we carried out our first safety embryo collection the same day. We collected on that occasion 3 embryos in the morning and 17 others in the evening, for a total of 20 good
embryos. As we carried out our safety embryo collections, we decreased the interval between the two collections so that we kept only the time of embryo research as a waiting period with positive results. We then came to the conclusion that the fact of finding embryos was certainly not always a question of time.

At the beginning, we targeted cows with a long slender uterus as being most often observed candidates in our population prone to give embryos in the second embryo collection. But this too empiric criterion was withdrawn after several cows with small uteruses, including some heifers, had once again given embryos in the safety embryo collection. We also withdrew the criterion “at least one good embryo” after at least 2 donors, which usually gave good embryos, had been successfully collected a second time whereas the result of their first collection was totally negative (No embryo and no oocyte).

Here are some results on cases that we documented in 2005 and 2006:

- Total safety embryo collections documented: 21 collections
- First embryo collection with at least one good embryo: 19 collections
- Average of embryos-oocytes per first embryo collection: (out of 21 collections): 3.62 embryos
- Number of safety embryo collections with a positive result: 12 collections (57%)
- Average number of embryos-oocytes found during safety embryo collections:
  - 3 embryos-oocytes out of 21 collections
  - 5.2 embryos-oocytes out of the collections with positive results

***Out of those 21 cases, the largest number of embryos-oocytes found was 21

***Out of the two embryo collections with a negative result at first, one donor gave 5 oocytes again and the other, one good embryo.

After those results, we decided to carry out a study during which we would collect from all our donors on two occasions. Here is the method we followed:

- Standard embryo collection with 650 ml PBS (½ in each horn)
- Return to first horn and we collect a second time with 650 ml PBS in a second beaker, therefore irrigating an extra 325 ml in each horn.
- Filter both beakers using different filters.

To our great surprise, here are the results we obtained:

- Number of embryo collections in the study: 192 collections
- Average number of embryos-oocytes collected in first embryo collection: 11.7 embryos+oocytes
- Average number of embryos-oocytes collected in the second embryo collection (All donors included): 1.88 embryos+oocytes
- Number of embryo collections with a positive result in second collection: 107 collections (56% of the collections)
- Average number of embryos-oocytes collected in second collection from donors with a positive result: 3.38 embryos-oocytes

In the second part of this home study, we questioned whether the fact of coming back in the horn was an important factor or if it was simply the fact that we collected with more PBS volume that allowed us to obtain more embryos. We therefore used the following protocol for this second part:

- Right horn embryo collection with the entire 650 ml but after 325 ml, we collect the PBS in a second beaker in order to see at which point the embryos were recovered.
- Left horn embryo collection using the same procedure as the right.
- Filter both beakers using two different filters.
The results obtained using this second method, which I nicknamed “en continu” (continuum), would deliver similar results. We therefore came to the conclusion that the fact of recovering more embryos was due to better irrigation of the uterine horns by using larger volumes of embryo collection liquid. We then went further by using 1.5 litre PBS at the rate of 750 ml continuously in each horn with the use of a second beaker after 500 ml of liquid collected. Here are the results we obtained:

Addition of a third 650 ml:
- Number of embryo collections in the study: 56 collections
- Average number of embryos-oocytes collected during first two embryo collections: 11.55 embryos+oocytes
- Average number of embryos-oocytes collected during the third embryo collection (All donors): 1.54 embryos+oocytes
- Number of embryo collections with a positive result in the third collection: 25 (42%)
- Average number of embryos-oocytes collected from donors with a positive result: 3.44 embryos+oocytes

At that moment, the number of embryos in the last 250 ml became a lot lower for my partners but remained fairly high in my case. However, it should be noted that during the last 250 ml of each horn, I increased the volume in each horn to 45-60 ml, doubling the PBS volume. Moreover, the following is a compilation of my last 40 embryo collections with 1.5 litres of PBS:
- Total donors collected with 1.5 litres: 40 donors
- Average number of embryos-oocytes after 1 litre: 7.45 embryos-oocytes
- Number of extra embryos-oocytes in the 500 ml: 1.95 embryos-oocytes
- Total donors having given embryos-oocytes in the last 500 ml: 27 (67.5%)

*** Out of the 40, 4 required a second embryo collection with 1 extra litre. They all gave embryos: Average of 10.35 embryos-oocytes during those second embryo collections.

*** 3 heifers did not give anything in the last 500 ml

*** 1 jersey cow gave one good embryo in the last 500 ml

We must come to the conclusion that we don’t know what causes the difference between donors delivering all their embryos to the first collection and those which require a second collection to succeed. However, we probably underestimated the presence of a physical interaction between the uterus and the embryo 7 days after ovulation. The presence of folds in the uterus most probably affects the embryos coming out of the uterus. Is there an existing valve where the uterus and oviduct meet in some cows and, if so, what mechanism provokes its opening? Is time an important factor? We observed that some donors tend to require automatically a second embryo collection in order to obtain most embryos. Also, we seem to have observed a certain family tendency. It would seem that in some donors, the fact of carrying out the embryo collection a little later (12-24 hrs) would help obtain embryos. However, all these questions and our attempts to explain them are simply hypothetical. Only our donors know the real answers.
EPIDEMIOLOGY OF EMBRYONIC MORTALITY IN CATTLE;
PRACTICAL IMPLICATIONS FOR AI AND EMBRYO PRODUCTION

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Introduction

Since the early work of Ayalon (1978) showing their critical impact on cow fertility, a lot of knowledge has been raised on the different types of embryonic mortality, their frequencies and the underlying mechanisms that lead to embryo or fetal losses at different stages of pregnancy. Although still incomplete in many ways, this information is very critical today as it has a lot of implications for field control of fertility and for the use and improvements of assisted reproductive technologies (ART) especially embryo transfer and in vitro embryo production. More recent developments and interests are associated to its use in the study of genes influencing fertility and for the improvement of the phenotypic information which is the basis and today the most important bottleneck in the implementation of genomic selection (Humblot et al., 2008a).

Methods of Evaluation and Frequencies of Pregnancy failures

The respective frequencies of the different types of pregnancy failures have been estimated first from embryo collection by uterine flushing performed at various stages following AI in slaughtered animals (Ayalon, 1978). This first work together with numerous studies based on the same methodology gathered more recently by Santos (2004) demonstrated that in most of the cases, fertilization rates are high and over 80%, both in beef and dairy cows, suggesting that embryonic death is responsible for most of the reproductive wastage following insemination in cattle (Zavy 1994, Humblot 1999). Due to the timing of the production and activity of antiluteolytic signals, it has been shown that very early embryonic mortality (EEM) before time of implantation (before day 14-16) do not induce any prolongation of luteal function (Northey and French, 1980, Humblot and Dalla Porta, 1984) and are followed by ovulation and (if observed) return in estrus in a range of 21-24 days after AI. Since this has been demonstrated, the relative frequencies of early embryonic mortality and later pregnancy losses could be estimated from larger groups of animals by associating systematic registration of return in estrus and progesterone (P4) measurements 21-24 days after AI (Humblot 2001, Santos et al., 2004, Grimard et al., 2006). Although not completely based on the same information, all types of studies demonstrated the major importance of early embryonic mortality representing both for beef cows and dairy cows 75% of total pregnancy failures (or 30%-35% of total AI’s). This percentage remains stable in beef breeds whereas more recent studies performed in lactating dairy cows evidenced a strong rise in the frequency of such failures which is nowadays close to 40% of total AI’s (Ponsart et al., 2007). Depending on studies, time and methods of evaluation, 10 to 20% of AI’s are followed by late embryonic and fetal losses inducing a prolongation of luteal function (Humblot 2001, Santos et al., 2004, Grimard et al., 2006). Pregnancy Specific Protein B (PSPB) and Pregnancy Associated Glycoprotein (PAG) measurements (Sasser et al., 1986, Zoli et al., 1991) have brought some new insight in the diagnosis and characterization of LEM allowing to distinguish two subgroups of cows among those with prolonged luteal function (Humblot et al., 1988) that helps to identify the sequence of specific effects that may affect embryonic development. The same methodology associating return in estrus and P4 measurements was applied together with measurements of Pregnancy Specific Proteins to investigate the frequencies of embryonic mortality after embryo transfer of in vivo or in vitro produced embryos (Vasquez et al., 1995, Chavat-Palmer et al., 2006, Van Wagendorp-de-Leeuw 2006). These studies shown that the frequency of both early and late embryonic mortality were higher in embryos issued from ART, especially for those issued from different in vitro production systems.
and nuclear transfer when compared to in vivo produced counterparts. Recently, Silva et al. (2008) reported also higher rates of EEM and LEM after transfer of half embryos than after transfer of whole embryos (respectively 62% vs 27% EEM, 16% vs 4% LEM). Additional losses at late stages of pregnancy were reported too from many studies dealing with in vitro produced embryos and nuclear transfer embryos (Greve et al., 1993, Horta et al., 1993, Farin and Farin 1995, Heyman et al., 2002, Renard et al., 2002, Ponsart et al., 2004, Panarace et al., 2007).

**Physio-pathological situations associated with Embryonic Mortality**

- **Influence of follicular growth and oocyte quality on early embryonic development**

There is now a lot of information in the literature that demonstrates the critical roles of oocyte quality and function at various stages of growth and meiosis in determining very early embryonic development (Sirard 2001, Sirard et al., 2006, Dieleman et al., 2002). It has been shown for some time that specific genes expressed during oocyte growth affects directly very early stages of embryonic development. Knock out experiments performed initially in mice demonstrated their critical role in embryonic development and there is now evidence for their presence and role in the bovine (Dean 2002, Pennetier et al., 2004, 2006). In addition to these specific genes, the role of many genes involved in the control of multiple functions within the oocyte and the embryo have been reported (Wrenzycki et al., 2005). The pattern of expression of the genes and accumulation of RNA transcripts during oocyte growth and maturation is really essential for early embryonic development as RNA’s and proteins accumulated within the oocyte at these early stages are used by the young embryo before the activation of his own genome (Hyttel et al., 2005). On top of this, the oocyte brings components of the developing nucleolus which are necessary for rebuilding the RNA machinery and expression of the embryonic genome (Hyttel et al., 2005). All those consequences of oocyte quality and function on early embryonic development have very strong implications for ART technologies and more generally for the control of fertility and probably explain the effects of many factors on fertility and embryo production efficiency. Due to the interactions developed between the oocytes and the follicles in which they are growing and getting mature, all factors influencing ovarian steroid production and follicular growth characteristics (number, size, speed of growth at different stages…) can potentially affect embryonic mortality rates. Such effects have been illustrated for instance by the positive correlation found between in vitro embryonic development and aromatase activity of the preovulatory follicles from which oocytes were issued (Driancourt and Mermillod, 1998) and by the fact that high embryonic mortality has been associated to prolonged dominance of the preovulatory follicle (Mihm et al., 1994). The negative effect of prolonged dominance on subsequent embryonic development is very consistent with the results of studies showing a lower fertility when cows have two waves of follicle growth instead of three (Townson et al., 2002) or when cows were submitted to long progestagen treatment (more than 12 days) in oestrus synchronization trials (Chupin et al., 1974). Similarly, beef cows which maintained dominance by a large and persistent dominant follicle before the LH surge had a reduced conception rate when compared to those with smaller and quick growing pre ovulatory follicles (Breuel et al., 1993). The consequences of prolonged dominance on oocyte quality have been shown to affect embryo mortality before the 16 cell stage (Ahmad et al., 1995) and thus may be the main source of repeat breeding. This information is very consistent too with the results of trials aiming at reducing and controlling the last phase of the cycle through a combined use of GnRH and PGF2 alpha allowing a significant reduction of embryonic mortality in repeat breeders (Humblot and Thibier, 1981, Thibier et al., 1985). On the contrary, lowering ovulatory follicle size by shortening too much the interval from follicle deviation to ovulation may have unfavorable effects as this was shown to increase the incidence of short luteal phase and reduces pregnancy rates (Vasconcelos et al., 2001, Santos et al., 2004).
Interactions between the early embryo and reproductive tract

Other mechanisms may be involved during early pregnancy that may lead to normal development or induce embryo mortality through alterations of interactions of the young embryo with the maternal genital tract.

It is known for a long time that progesterone regulates oviduct and uterine functions (Miller and Moore, 1976). As a consequence of this, it has been hypothesized that low progesterone concentrations following fertilization may induce a less favorable environment for the embryo. This hypothesis has been supported by results of studies in which naturally or induced low progesterone concentrations were associated to decreased fertility (Lamming and Royal, 1999) or increased embryonic mortality (Miller and Moore, 1976). Detrimental effects of an unfavorable environment inducing a slow growth of the embryo may be expressed some days later and associated to a low and/or late secretion of antiluteolytic signals thus leading to late embryo mortality and delayed luteolysis (Royal et al., 2000). However, despite the fact that insufficient progesterone secretion has been often cited as a cause of embryonic mortality, its real negative impact and the associated interest of post-AI progesterone supplementation and/or stimulation of progesterone secretion are still very controversial under temperate climatic conditions.

Effectively, the occurrence of low progesterone values has been reported to be extremely rare even in repeat breeder cows in which embryonic mortality is more likely to occur (Humblot and Thibier, 1981). From experiments during which embryos were flushed, Ayalon (1978) and Linares (1981) did not find any difference in progesterone concentrations between cows carrying normal, degenerated or retarded embryos. In addition, no relationship has been found between embryo survival after embryo transfer and progesterone concentrations measured the day of transfer (day 7) in recipients (Humblot et al., 1987). In very few experiments post AI progesterone supplementation or stimulation of progesterone secretion were shown to increase conception rates (Mann and Lamming, 1999, Mac Millan et al., 1986). However, in most studies when progesterone supplementation was used or progesterone secretion was stimulated by hCG or GnRH injections, treatments failed to reduce embryonic mortality rates (Phatak et al., 1986, Chenault, 1990, Lewis et al., 1990, Swanson and Young, 1990, Lajili et al., 1991, Tefera et al., 2001).

On the contrary to the lack of positive effects most often reported when administered post-AI, a better use of progesterone could be done by utilization of progesterone releasing devices prior to AI as it has been shown that such treatments constantly increases the ovulation rate, reduce the frequency of subsequent short luteal phase thus improving embryo survival (Santos et al., 2004).

Another possibility that may be an important source of embryonic mortality is that the reproductive tract may not fully recover following calving and so does not provide a suitable environment for early embryo development (Wathes et al., 2008). On top of pathological situations (incomplete uterine involution and endometritis that will not be considered here), there is now more clear evidence that deviating or altered immune reactions involved in uterine tissue regeneration and remodeling and can also be the source of embryonic mortality taking place by the time of maternal recognition of pregnancy (Wathes et al., 2008).

Factors influencing early and late embryonic mortality

The sources of variation of embryonic mortality have been studied both from experiments and studies designed to investigate the role of a single factor and more frequently from several multi-factorial epidemiological field surveys (Grimard et al., 2006, Freret et al., 2006, Ponsart et al., 2007). The previously described methodology (Humblot, 1999, 2001) including milk or blood samplings for progesterone measurements on Day of AI and 21 days later and PSPB assays and/or ultra sound examination was used to estimate the respective frequencies of EEM and LEM and their sources of variation. The results of these multi-factorial studies are summarized in Table 1 and will be discussed with results from former mono factorial experiments.
Table 1: Sources of variation of EEM and LEM according to multi-factorial studies. Only significant sources of variation after adjustment for other factors and issued from log-linear models are mentioned. (NS = non significant ; U.A. = under analysis)

<table>
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<tr>
<th>Source of variation</th>
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<td>Genetic Merit (G) / Breed (B)</td>
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<td>LEM</td>
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<td>Calving conditions</td>
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<td>Behavioral signs used for AI decision</td>
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<td>Moment of AI (interval from heat detection)</td>
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<td>Restraining conditions</td>
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<td>Milk Production</td>
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<td>High BCS</td>
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<td>Transition period management</td>
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1) from Humblot, 2001, Grimard et al., 2006 ; 2) from “FERTILIA”, UNCEIA, unpublished data (material and methods described in Freret et al., 2006, Ponsart et al., 2007) ; 3) from Michel et al., 2003

Genetics

From several models, genetic merit within the Holstein breed were found on EEM, indicating that cows with high milk indexes are less fertile than females with lower genetic merit after adjustment for milk production (Table 1). Breed effects have been observed also both on EEM and LEM. Prim Holstein females presenting lower fertility performances than cows from the Normande breed (12 and 15 points of EEM and LEM respectively). This fits well with the phenotypic differences reported between those two breeds in terms of AI efficiency (Barbat et al., 2005). Those results are very consistent with the information obtained in different countries illustrating clearly that genetics and especially selection for milk production affects fertility (Boichard et al., 1998, 2002, Royal et al., 2000, Lucy, 2001, Bousquet et al., 2004). Negative genetic correlations exists between milk production traits and fertility variables which explains most of the negative phenotypic evolution reported for fertility traits during the past 20 years (Barbat et al., 2005, Ponsart et al., 2007). Different mechanisms including interactions with metabolic processes such as energy deficit and negative energy balance which are more likely to occur in high producing cows during the post partum period may explain the relationships mentioned above. However, part of those effects may be due to processes that are not related to the effects of milk production. Additive effects of the genetic index and of the level of milk production have been illustrated (Humblot, 2001) showing that the genetic index of the cows influenced fertility whatever the level of milk production (Figure 1). In addition, effects of the genetic index mainly affected EEM whereas milk production level was mainly associated to LEM (Humblot, 2001, Grimard et al., 2006). This is very consistent with earlier findings (Humblot and Denis, 1986, Boichard and Manfredi, 1995), or from more recent studies combining genotyping and phenotyping (Humblot et al., 2008b) showing that paternal effects on daughter
fertility are expressed mainly on EEM and not on LEM. It is likely that such genetic effects on EEM are mediated through changes in follicular growth and/or oocyte growth and maturation susceptible to influence oocyte quality. It is clear that proper selection based on the use of pertinent genomic markers may help to reverse the negative trends in reproductive efficiency observed those last years in most dairy breeds (Barbat et al., 2008). As an example, the precision (coefficient of determination) used for genetic selection for fertility based on an early genomic index will be multiplied by 3 when compared to the previous estimation based on quantitative genetics (0.6 instead of 0.2) and will be higher than the precision (0.45) obtained in the past after progeny testing (S. Fritz, personal communication).

Figure 1: Additive effects of genetic merit (milk index < or > 27) and daily milk production at peak of lactation on fertility rates in Prim Holstein cows (from Humblot, 2001). For each variable the upper class represents the top 25% of the population.

Bull

The bull effect has not been studied from previous epidemiological studies 1 and 3 and has been found not significant in study 2 from multifactorial models. However, the number of AI’s per bull was relatively low and most of the time, significant differences between males in terms of non return rates have been reported since AI has been developed (Salisbury et al., 1952). The analysis of the distribution of non return rates with time suggested that the bull has an effect on fertilization rates and early embryonic mortality (Bearden et al., 1956, Humblot, 1986). Bull’s differences in terms of embryonic development have been further documented by studies in which differences in embryo production were observed from donor cows following superovulation (Humblot et al., 1986, Manciaux et al., 1997). These observations have been completed by in vitro studies showing differences between bulls in terms of fertilization rates and rates of development up to the blastocyst stage which are correlated with field non return rates (Marquant Leguienne et al., 1990, Marquant Leguienne et Humblot, 1992). The lower ability of some bulls to induce early embryonic development may be associated to differences in speed of development. Such a relationship between speed of development and embryo survival has been first documented in rodents (Warner et al., 1984) and then confirmed in cattle by in vitro studies showing a positive relationship between time of first cleavage and embryonic death by Day 8 (Lonergan et al., 1999) and a bull effect on the timing of the different phases of first cleavage (Comizzoli et al., 1998). More recently a very important bull effect was found on rate of development of reconstituted embryos following the cloning of blastomeres issued from biopsied embryos of different paternal origin (Figure 2; Lebourhis and Humblot., 2008) suggesting that remnant effects may exists following activation of the egg. Taken as a whole, this information illustrates very well strong effects of the bull on early embryonic development that could be mediated through DNA integrity (Rodriguez Martinez, 2007) and interactions of sperm cells with the egg activation processes that involves Ca++ content and release following fertilization (Perry et al.,
2000, Ozil and Huneau, 2001, Thibault, 2001). On the contrary, differences between individual bulls in terms of late embryonic mortality have been reported to be very low (Humblot, 1986). However, when estimated, the sire has been shown to have an effect on foetal losses in cattle (Lopez Gatius et al., 2002).

![Figure 2: Survival rates of biopsied embryos issued from different bulls and number of reconstituted blastocyst following cloning of individual blastomeres (from Lebourhis and Humblot, 2008).](image)

**AI Conditions**

The relationships between heat detection, AI conditions and embryonic mortality were mainly investigated in study 2 (Table 1). Behavioral signs of oestrus used by the farmer to call the AI technician were related to EEM (Table 2). The frequency of EEM was significantly increased when other signs of oestrus than standing heat or mounting activity (alone or in association) were used to call the AI technician. Time of AI (relative to heat detection) as well as restraint quality was associated to EEM. Both early (up to 6 hours) and late insemination (later than 24 hours) increased the incidence of EEM, but also of LEM (Figure 3).

**Table 2: Relationships between the behavioral sign used by the farmer to call the AI technician and incidence of EEM (from “FERTILIA”, UNCEIA, unpublished data); (*) Significantly different from reference (OR =1).**

<table>
<thead>
<tr>
<th>Behavioral sign</th>
<th>Incidence of EEM (%)</th>
<th>OR</th>
<th>Low CI (95%)</th>
<th>High CI (95%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Standing heat</td>
<td>38.5</td>
<td>0.842</td>
<td>0.661</td>
<td>1.073</td>
<td>1153</td>
</tr>
<tr>
<td>Single mounting activity</td>
<td>35.2</td>
<td>0.969</td>
<td>0.732</td>
<td>1.284</td>
<td>533</td>
</tr>
<tr>
<td>Single sexual behaviour</td>
<td>30.7</td>
<td>1.187</td>
<td>0.668</td>
<td>2.109</td>
<td>69</td>
</tr>
<tr>
<td>Other sign : Mucus + nervous + beugl + calendar + production variation + heat detection tool…</td>
<td>(*)45.8</td>
<td>0.624</td>
<td>0.453</td>
<td>0.861</td>
<td>282</td>
</tr>
<tr>
<td>Multiple signs including standing</td>
<td>34.5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple signs including mounting</td>
<td>40.6</td>
<td>0.771</td>
<td>0.531</td>
<td>1.118</td>
<td>171</td>
</tr>
<tr>
<td>Multiple signs including sexual behaviour</td>
<td>44.3</td>
<td>0.662</td>
<td>0.344</td>
<td>1.274</td>
<td>42</td>
</tr>
<tr>
<td>Other multiple signs</td>
<td>(*)48.9</td>
<td>0.550</td>
<td>0.332</td>
<td>0.911</td>
<td>75</td>
</tr>
</tbody>
</table>
Places and means of restraint were grouped together according to their effect on lack of fertilization (NF) and EEM rate and classified as UFR= unfavourable, IR= intermediate and FR= favourable type of restraint. Unfavourable restraint comprised box with corridor or cubicle without cow being tied-up or cow inseminated in the milking parlour. Intermediate conditions were those in which AI was performed in loose housing conditions or in a box, with head-locking restrainer, or box with a barrier. Favourable restraining conditions were identified as box with head-locking restrainer or corridor with restraining bar or tie stall barn or cubicle with cow being tied-up by the farmer or cow tied-up with a rope or a halter. Lack of fertilization and EEM rate was globally 36.8%, and was influenced by the type of restraint: UFR, 40.7% (n=398), IR, 37.1% (n=2961) and FR, 32.2% (n=488), (Figure 4) and by the quality of the restraining method as judged by the technician at time of AI: good, 34.8% (n=2188), nearly good, 38.9% (n=1675), bad/nearly bad, 37.2% (n=277) (Freret et al., 2007).

Figure 4: Percentages of cows with a good quality of restraint as judged by the AI technician and effect of the restraining conditions on the frequency of lack of fertilization and early embryonic mortality (NF-EED) (from “FERTILIA” : Freret et al., 2007, Ponsart et al.,2007)

Not so much data exists in the literature on the effects of those factors related to AI conditions. It may be speculated that when conditions are unfavorable (i.e. in case of lack of clear signs of estrus, bad restraining conditions, and bad timing of AI) these different factors may induce a lack of fertilization or fertilization at a time which is not optimum for oocyte quality and subsequent embryo development.
The time of detecting estrus during the day influenced LEM. Incidence of LEM was increased when detection occurred during feeding compared to a period of inactivity (48 vs 34%, p<0.05). The incidence of LEM was increased also when AI was made on induced estrus when compared to natural heat (44 vs 35.5%, p<0.05) (from “FERTILIA”, UNCEIA, unpublished data). Despite negative progesterone concentrations at time of AI were measured in those cows, a possible confusion with bad timing of AI and cyclic irregularities may exist for those sources of variation of LEM.

**Milk production, Nutrition and Energy Balance**

Milk production of cows was a major factor observed in the 3 different studies, and found significant even after adjustment for body condition score (study 1). Milk production at the peak of lactation was associated with EEM in study 1 and with LEM in 2 studies, whereas milk production at AI was related to EEM in study 3. These factors are linked to the energy balance and BCS changes after calving and EEM was significantly reduced when AI were performed a long time after calving (>= 90 d vs <70 d). More recently a link with the duration of the pre-calving transition period was found (Studies 2 and 3). EEM was decreased in cows with dry periods lasting from 55 to 75 days compared with shorter time intervals (<55 d; study 3). This is in agreement with the results of other studies showing very clear relationships between indicators of Negative Energy Balance (NEB) such as protein milk content, low BCS / high BCS losses and embryonic mortality (Ponsart et al., 2007). In former studies (Wiltbank et al., 1964, Dunn et al., 1969, Francos et al., 1977), an induced reduction in energy supply/intake has been associated with lower fertility and higher embryonic mortality. Similarly, in beef heifers, a reduction from 2.8 maintenance to 0.8 maintenance energy needs was associated to lower embryonic survival with most of embryonic losses occurring before Day 14 following fertilization (Dunne et al., 1999). More recently, specific unfavorable effects of NEB on follicular growth and oocyte quality have been reported from experimental work (Snijders et al., 2000) and have been related to low IGF and low insulin concentrations and or low sensitivity of the ovary to insulin (see Humblot et al., 2008c for review). In most of studies, a higher frequency of abnormal progesterone patterns following ovulation were reported especially in High genetic merit cows (Pushpakumara et al., 1999, Lucy and Crooker, 1999, Ponsart et al., 2007, 2008). The results of all these studies fits very well with the above mentioned mechanisms by which an unfavorable follicular environment can affect oocyte quality and subsequent embryonic development. However, several studies have shown that the conditions and nutritional status that are optimum for follicular growth are not exactly the same as those required for oocyte quality and embryonic development. Most of this information has been obtained in overfed animals in which, on the contrary to what was mentioned before, insulin levels were increased under regimes inducing a quick growth (Freret et al., 2006b) or in animals that have been overfed for a long time (Humblot et al., 1998, Bage et al., 2002). This may lead, as recently shown by Garnsworthy et al. (2009), and contrary to most nutritional recommendations that avoids nutritional changes, to apply sequential regimes that first increase insulin concentrations to stimulate follicular growth and then decrease insulin for the sake of embryonic development. There is now evidence that nutritional effects may affect too the quality of interactions between the embryo and the uterus as it was recently shown that cows in severe negative energy balance had altered proportions of immune cells within the endometrium and that several components of the IGF system were involved in the control of tissue remodeling and repair within the uterus (Wathes et al., 2008). In the same paper, the authors report up regulation of immune related genes in cows with severe negative energy balance when compared to their counterparts and many genes involved in protein modification and collagen catabolism (especially metallo-proteinases were also regulated by negative energy balance). Such mechanisms may explain the relationships found between the BCS status of cows by time of calving and the occurrence of late embryonic mortality reported by Humblot (2001) and Grimard et al. (2006).
Season, Temperature and Heat Stress

Many studies have shown that conception rates are reduced (10 to 15%) when the external temperature is elevated at time of insemination (Francos and Mayer, 1983, Badinga et al., 1985) and both rectal and uterine temperature are negatively correlated with conception rates (Ulberg and Burfening, 1967, Thatcher, 1974). Exposure of animals to high temperature may increase embryonic mortality rates through different mechanisms but most of the studies stressed out effects of temperature on early embryonic mortality (Ulberg and Burfening, 1967, Wise et al., 1988, Geisert et al., 1988). More recent studies have shown that the quality of the oocyte could be impaired in heat stressed cows thus resulting in poor early embryonic development (Hansen, 2002) and poor developmental competence (Al-Katanani et al., 2002). Follicular growth and steroidogenesis have been shown to be affected as well by high temperatures (Wolfenson et al., 1997, Zeron et al., 2001, Roth et al., 2001a). Part of those effects on oocyte quality may be due to a previous alteration of the quality of small follicles and subsequent oocyte growth during previous weeks/months (Roth et al., 2001b) resulting in modifications at time of final growth and dominance (Badinga et al., 1993). This may explain medium term effects of heat stress on embryo quality such as those reported in superovulated donor females (Ealy et al., 1993) even when collections were made one or two months after the peak of heat stress (Figure 5, Ponsart et al., 2005).

Figure 5: Deviation of embryo viability from embryos collected after superovulation (% collections with one viable embryo at least) following heat stress (july-august); differences between each month of collection and the annual regional mean in 2003 in three regions of France (From Ponsart et al., 2005, total number of flushings = 2270).

Short term or mid term effects on the oocyte are probably the main mechanisms by which temperature affects early embryo development as Drost et al. (1999) demonstrated that transfer of in vivo produced embryos from cows exposed to thermoneutral temperatures increased pregnancy rates in heat stressed recipients cows when compared to results following transfer of embryos issued from heat stressed cows. The importance of such mechanisms in relation to oocyte function is further supported by the fact that the sensitivity of the embryos to heat stress is observed mainly during the first week of development (Putney et al., 1988a, Barros et al., 1992, Wolfenson et al., 2000).

However, direct effects of temperature affecting the embryo itself at a later stage can’t be excluded and are likely to occur under high temperature environment especially in cows. Elevated body temperature increased late embryonic mortality in cows with an acute infection of the mammary gland (Fournier and Humblot, 1989) and in heifers receiving interferon alpha 1 injections (Barros et al., 1992). These effects
could be mediated through an increased sensitivity of the CL to PGF2alpha (Putney et al., 1988b) and/or to a lower antiluteolytic signal by the time of implantation. High rates of late embryonic mortality were reported under tropical or subtropical conditions especially during the wet and warm season (Tillard et al., 1999) and the frequency of pregnancy losses by this time was doubled (over 20%) when compared to those obtained during the dry season or to those usually reported in Europe. Under French conditions, exceptions such as those mentioned above which were observed during year 2003 may exists but summer usually does not seem to affect negatively embryonic mortality as this season was shown recently to be associated to lower rates of EEM and LEM in 2 studies (Table 1) which corroborates earlier results from Fournier and Humblot (1989).

Finally, due to their decreased ability to control body temperature resulting from the higher metabolic rate associated to milk production (Berman et al., 1985) that induces higher average basal temperature (Sartori et al., 2002), lactating cows may be more sensitive to heat stress than heifers. This may explain the higher rate of collections without any transferable embryo observed in heat stress donor cows when compared to contemporary heifers (Ponsart et al., 2005).

**Age and factors related to calving**

As frequently reported before, EEM was reduced in primiparous cows and in females with an easy calving (table 1). This is in agreement with studies based on non return in estrus after AI (Steffan and Humblot, 1985, Boichard and Manfredi, 1995). However, specific effects of age on LEM have been also documented from the analysis of return in estrus (Bar Anan et al., 1979, Humblot and Denis 1986) and from studies combining those with progesterone measurements (Ball, 1978, Fournier and Humblot, 1989).

**Conclusions**

A lot of information has been raised showing the impact of events preceding ovulation on early embryonic mortality in relation with follicular growth and oocyte quality. Most epidemiological studies run recently show that factors influencing pregnancy and embryonic mortality rates can impact follicular growth and oocyte quality a long time before insemination takes place. This information should lead to develop successful practices to counterbalance the effects of factors affecting negatively, alone or in combination, fertility and embryonic mortality, such as high milk production, Negative Energy Balance and heat stress. Considering the post-pone effects of those factors resulting in poor oocyte quality during the post partum period and for some of them that takes place prior to calving, preventive measures should be more efficient to control fertility and in reducing embryonic mortality than late compensatory treatments. Part of this information could be used as well to develop new strategies to improve the efficiency of embryo related assisted reproductive techniques.

**References**


THE DIAGNOSIS AND TREATMENT OF POSTPARTUM ENDOMETRITIS
IN DAIRY CATTLE

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Introduction

Reproductive efficiency is the cornerstone of modern dairy herd management. Cows must calve at intervals that maximize individual cow lifetime production and profitability of the dairy herd [1]. Postpartum endometritis is one of the many causes of reproductive inefficiency in a dairy herd. With the advent of new diagnostic techniques and computerized analysis of herd reproductive performance endometritis has been shown to result in considerable economic loss to the dairy industry. The impact on fertility is usually translated into an increase in days open and an increase in services per conception [2]. Other costs may be realized through premature culling [2, 3, 4], disease treatment, and slowed genetic gain because of fewer calves being born.

Postpartum Uterine Physiology and Pathogenesis

Most uterine infections are associated with parturition. During the first 6 weeks after calving the uterus undergoes the normal process of sloughing and regeneration of the endometrium known as involution [5]. Nearly all cows have bacterial infection of the uterus in the first 2 weeks postpartum [6]. The perpetuation of infection depends upon the presence of substrate, the degree of bacterial contamination and the various uterine defense mechanisms [7]. The immune system of the dairy cow is suppressed during the last weeks of gestation and up to the first 3 weeks of the postpartum period [8] largely due to the effects of elevated progesterone and estrogen concentrations [4]. Both are capable of modifying the expression of hormone receptors in immune cells and both, especially progesterone, reduce the secretion of prostaglandins from stromal cells stimulated by gram-negative cell wall lipopolysaccharide (LPS). Furthermore, metabolic substances such as insulin-like growth factor 1 (IGF1) and numerous other proteins have been shown to have immunomodulatory properties in the endometrium [4]. During and around the time of parturition the physical barriers to genital infection: the vulva, vagina and cervix are compromised enabling entry of bacteria into the uterus. Throughout the immediate postpartum period the pattern of infection, clearance and re-infection occurs repeatedly [5, 9]. A variety of bacterial species may be cultured from the uterus during the postpartum period; however, virulent strains of Arcanobacter pyogenes, capable of causing disease alone or in combination with the gram-negative anaerobes Fusobacterium necrophorum and Prevotella melaninogenicus, are most frequently encountered [9]. Escherichia coli may also be found with the uterus, but tends to be more prevalent earlier in the postpartum period [5, 9]. Infection with E. coli may predispose to infection with other bacterial species, particularly A. pyogenes [4, 10, 11]. A. Pyogenes is responsible for the most severe damage to the endometrium and to date all strains isolated from the uterus have been found to produce a cytotoxin pyolysin capable of destroying endometrial cells [4]. It is well known that a synergistic relationship exists between A. pyogenes and the Fusobacterium and Prevotella species. Arcanobacter pyogenes produces a growth factor for F. necrophorum; F. necrophorum produces a leukotoxin; B. melaninogenicus produces a substance that prevents bacterial phagocytosis; and A. pyogenes has been shown to decrease neutrophil chemotaxis and phagocytosis [9].
Bovine herpesvirus 4 (BHV-4) is the only virus associated with uterine disease in cattle. Latency may be established in macrophages with virus multiplication occurring within endometrial epithelial and stromal cells ultimately resulting in their death. Research to date, has shown that BHV-4 must act in concert with other bacteria that cause uterine disease. Virus activation seems to be dependant upon bacterial endometritis-induced release of prostaglandin E (PGE) followed by PGE and *E. coli* LPS stimulated viral replication. Control of BHV-4 genital infections may become an important means of reducing the impact of endometritis in the future [4].

In ewes it has been shown that LPS suppresses GnRH release, LH secretion and pituitary sensitivity to GnRH resulting in a failure to ovulate. Lipopolysaccharide infusion into the uterus of cattle has resulted in similar findings although the concentration of LPS required to produce an effect needs to be substantial. Follicle stimulating hormone appears to be unaffected by these changes; therefore, follicular waves continue to occur as in normal animals [4].

**Defining Endometritis**

The word “metritis” is often misused by researchers, veterinarians, and hence lay persons when referring to a number of inflammatory conditions of the uterus including normal physiologic processes. However, it must be stressed that metritis differs greatly from endometritis both histologically and clinically [8]. Metritis occurs most frequently between the first week and second week post-calving and is severe inflammation involving the endometrium, myometrium and possibly the serosal layers. Metritis is characterized by an enlarged uterus and watery brown fluid or viscous grayish-white purulent uterine discharge [4]. Most cows with metritis exhibit clinical illness due to the absorption of harmful substances produced by bacteria. “Endometritis”, on the other hand, is inflammation involving only the endometrium and tends to occur much later in the postpartum period. To avoid confusion with normal uterine involution the term endometritis should be reserved for those cases of uterine disease or impaired involution which persist beyond 3 weeks postpartum. Endometritis is not associated with obvious clinical illness and is usually due to persistence of a mild infection that was not cleared during involution [2]. A purulent or muco-purulent discharge may be evident at the vulva or cervix associated with an influx of inflammatory cells, mostly neutrophils, into the endometrium [4], or there may be no obvious clinical signs. The presence of purulent or mucopurulent mucus is significantly associated with the number and type of pathogenic bacteria present; in particular, *A. pyogenes*, *F. Necrophorum* and *Proteus sp*. Fetid odour, sometimes noted when examining purulent exudate, is associated with a greater acute phase protein response by the liver in response to pro-inflammatory cytokines released during tissue damage and bacterial infection. The likelihood of fetid odour is reported more commonly in cases of endometritis diagnosed at 21 verses 28 days and is associated with *A. pyogenes*, *E. coli*, non-hemolytic Streptococci and *Mannheimia haemolytica* [12].

Approximately 15-20% of dairy cattle will have clinical endometritis with a further 30% experiencing subclinical disease [4]. Clinical endometritis may be thought of as overt signs of disease visible to the naked eye. Because endometritis is so easily confused with normal involution a great deal of effort has been spent determining what is disease and what is normal. Only within the past decade has there been an effort to determine disease status based on retrospective evaluation of the effect on reproductive performance. For the most part, endometritis may be thought of as compromised uterine involution. As such, clinical signs normally associated with endometritis must be considered in light of the number of days since calving. This has resulted in the current definition of clinical endometritis as the presence of a purulent uterine discharge visible at the vulva 21 days or more postpartum, or a mucopurulent discharge detectable within the vagina after 26 days postpartum [4].

Endometrial inflammation must also be considered at the histologic level. Disruption of the epithelium, increased blood flow, edema and an influx of inflammatory cells, mostly neutrophils and lymphocytes, occurs in all cases [8]. Many affected animals do not exhibit classical uterine discharge, but
do have evidence of inflammation based on cytological examination and consequent impaired reproductive performance compared to their non-affected herdmates known as subclinical endometritis [13, 14, 15]. Neutrophils are the first and most significant inflammatory cell involved in endometritis, but of course are also involved in the involution process. To facilitate diagnosis, threshold numbers of neutrophils have been determined based on the time after parturition and evidence of reduction in reproductive performance [14, 15]. For the most part, subclinical endometritis may be thought of a milder form of clinical disease; however, it is doubtful that its impact on reproductive performance is any less severe.

One of the most significant obstacles in diagnosing endometritis has been to establish the most appropriate time period to examine cows for evidence of disease. The cytological criteria for the diagnosis of subclinical endometritis continue to be refined, but so far the stage of the postpartum period when samples are obtained seems to be the most significant variable. All cows have some degree of uterine inflammation up to 30 to 35 days postpartum; therefore, it is imperative that measures of reproductive performance are used to evaluate diagnostic and ultimately treatment protocols. Reproductive performance parameters have included pregnancy rate [2,14,16-18], interval from calving to first service [2, 14, 17, 18], interval from calving to pregnancy [2, 14, 17,18], first service conception rate [2, 14, 17, 18], services per pregnancy [2, 16, 17] and overall conception rate [14, 18].

Diagnosis

Transrectal palpation of the uterus was once the most popular technique used to diagnose endometritis [2, 16]. Enlarged uterine horn(s), asymmetry of the uterine horns, thickness of the endometrium and the presence of a palpable uterine lumen and/or palpable fluid within the lumen were suggestive of abnormal uterine involution or endometritis [2] based upon a poorly defined description of normal. Transrectal palpation is now considered to be an insensitive method of diagnosis [2, 16]. Simply too many cases are missed when palpation is used as the only diagnostic test.

Direct diagnostic techniques such as uterine biopsy and bacterial culture have been considered by some to be the gold-standard tests for endometritis. Neither of these techniques are widely used owing to a number of factors; notwithstanding, neither are cow-side tests, nor are they very practical. Most infections persisting beyond 3 weeks after calving are believed to principally involve strains of A. pyogenes; and a single uterine biopsy is not as representative of the entire uterus and may impair future reproductive performance [19]. Furthermore, histological evidence of inflammation determined by biopsy was only able to account for 3% of the variation in the number of days open [20].

Vaginoscopy is a very useful diagnostic technique. Compared to uterine culture the sensitivity of vaginoscopy was reported to be 59% verses only 22% for transrectal palpation [21]. By using vaginoscopy, more cows having an abnormal discharge can be identified; however, vaginoscopic examinations performed before 26 days postpartum can result in an increased number of false positives [2]. Using survival analysis to derive a case definition for clinical endometritis based on factors associated with increased time to pregnancy various diagnostic techniques including external inspection, vaginoscopy and palpation of the uterus and ovaries were compared. The study, one of the most powerful to date, involved 1865 cows in 27 herds examined once between 20 and 33 days postpartum [2, 8]. Only a purulent discharge visible externally or a cervical diameter exceeding 7.5 cm after 20 days postpartum, or a mucopurulent discharge visible after 26 days postpartum using vaginoscopy were useful for identifying cows with endometritis. None of the diagnostic criteria based on palpation of the uterus proved to be useful for predicting time to pregnancy. The overall prevalence of clinical endometritis in that study was 17%. Without vaginoscopy, 44% of the cases would have gone undiagnosed. Employing vaginoscopy before 26 days resulted in too many normal cows being incorrectly classified [2]. Other ways of detecting uterine discharge have been studied including the gloved hand [22] and the Metricheck device [23] and appear to be as efficacious as vaginoscopy. Unfortunately, even when used after 26 days postpartum vaginoscopy failed to identify all cows that were at risk of poor reproductive performance due to
Numerous cows experience an impairment of reproductive performance due to subclinical endometritis. The most ideal diagnostic test for endometritis should identify all cows at risk of impaired reproductive performance due to both clinical and subclinical endometritis, and do so in a timely fashion so that the appropriate treatment may be administered. To gain widespread acceptance this test must also be a simple and offer an immediate result.

Endometrial cytology, based on the presence of cellular evidence of inflammation, is an accepted way to evaluate uterine disease in cattle [3, 5, 9, 14]. Inflammatory cells may be recovered by either of two techniques: uterine lavage or cytobrush. Uterine lavage involves the infusion of 20 to 60 ml of 0.9% saline into the uterine lumen using a sterile plastic infusion pipette followed by gentle massage of the uterus before re-aspiration of the fluid through the same infusion pipette. Fluid samples are centrifuged, cellular debris is recovered and smeared onto a slide, and microscopic analysis is performed after staining with modified Wright-Giemsa stain [3, 15, 16, 24]. The cytobrush method employs a small brush attached to handle originally designed for human use [15, 24]. The handle may be trimmed to a length of 3 cm or less and threaded into a 65 cm long x 4 mm diameter stainless steel rod. A 5 to 6 mm diameter stainless steel tube 50 cm long protected by a plastic sanitary sleeve is used to guard the instrument before passage into the uterine lumen [14, 15]. Within the uterine body, the instrument is rotated to collect cellular material from the endometrial surface which is later rolled onto a slide and prepared for analysis as is done for lavage samples [14, 15]. Cytological assessments of the severity of inflammation are made by determining the percentage of neutrophils per 100 cells at 400X [14-16]. Threshold neutrophil percentages varying between 5 and >18% have been reported to be indicative of an endometritis-positive status when survival analysis was used to evaluate the rate at which cows became pregnant [14-16]. Greater threshold percentages were applicable to cows sampled earlier in the postpartum period, at 20 to 33 days [14] whereas, thresholds of 5%, >8% and >10% have been reported for cows sampled at 40 to 60, 28 to 41 and 34 to 47 days in milk, respectively [14-16]. Barlund et al. [15] showed that >8% neutrophils in samples collected from cows 28 to 41 days in milk using the cytobrush had a sensitivity of 12.9% and a specificity of 89.9% for pregnancy status at 150 days in milk. Samples collected from the same cows by lavage showed a sensitivity of 14.3% and a specificity of 84.0% using the >8% neutrophil threshold. The lavage and cytobrush outcomes were very highly correlated; however, the lavage technique was considered to be less reliable because of a loss of cell definition speculated to have been caused by the additional steps required to harvest the cellular material. Neither technique was very sensitive when evaluated with respect to 150-day pregnancy status attributed to a number of other factors that may affect pregnancy status, or because of spontaneous recovery [15]. However, cows with >8% neutrophils in cytobrush-acquired samples were shown to have a 24-day increase in median days open.

Intrauterine fluid has been shown to be associated with bacterial growth and impaired uterine involution [25]. The presence of uterine fluid in cows examined using ultrasound between 20 and 47 days in milk was associated with a 62 to 63 day increase in median days open compared to those having no fluid [14]. Both Kasimanickam et al. [14] and Barlund et al. [15] reported that the presence of fluid in the uterine lumen and positive endometrial cytology were effective for diagnosing endometritis, but the tests did not identify the same cows. Endometrial cytology identified cows having mainly a cellular response to inflammation whereas, ultrasound identified cows with impaired uterine clearance mechanisms. Using a 3 mm uterine luminal diameter as the threshold accumulation of uterine fluid and endometritis-positive status as the outcome Barlund et al. [15] reported sensitivities of 30.8% and 10%, and specificities of 92.8% and 93.3% when comparing evidence of fluid with cytobrush cytology and 150-day pregnancy status, respectively. Using a smaller threshold of 1 mm improved the sensitivity of the uterine fluid measurement, but at the expense of a nearly equal loss in specificity. According to their data the most ideal threshold luminal diameter was between 1 and 3 mm. When the results of cytobrush cytology (> 8% neutrophils) and > 3 mm luminal diameter were combined the sensitivity nearly doubled to 21.6 % for predicting pregnancy status at 150 days. This finding combined with a lack of agreement between the two tests (kappa = 0.25) provided more support for a two-disease syndrome. Therefore, it appears that there
are cows that have a cellular response to inflammation without fluid accumulation, and cows that have fluid accumulation or decreased uterine clearance with very few detectable inflammatory cells.

Endometrial thickness has long been associated with endometritis or impaired uterine involution. By ultrasound scanning the uterus and measuring the thickest portion of the endometrium Barlund et al. [15] reported that as a predictor of endometritis status the most appropriate measure was between 7 and 8 mm, but that it was not a sensitive or reliable predictor of pregnancy status at 150 days and compared very poorly with cytobrush cytology.

Ultrasound technology is useful for identifying some, but not all of the cows with endometritis. This is unfortunate as ultrasound equipment is becoming commonplace in most veterinary practices and has the advantage of an immediate diagnosis. Cytobrush cytology appears to be the most reliable diagnostic technique, especially for subclinical endometritis, but is not a cow-side test. Until there is new evidence to support the contrary, ultrasonographic assessment of uterine fluid and cytobrush cytology should be combined in research studies to obtain an accurate assessment of disease status. Ultrasonography and cytobrush cytology continue to be evaluated with a hope of perfecting our ability to diagnose economically relevant cases of endometritis. Vaginoscopy is also very amendable to routine on-farm use and is the simplest, most reliable and cost-effective way to diagnose clinical endometritis.

Treatment

Treatment regimes used in the past tended to lack scientific merit because of inadequate diagnostic techniques and poorly designed experiments. Notable flaws include a lack of understanding of normal postpartum uterine physiology and basing the cure rate on transrectal palpation findings rather than determining the impact on reproductive performance criteria. Currently supported treatment regimes include intramuscular injection of prostaglandin F$_{2\alpha}$ (PGF) and intrauterine infusion of antibiotics. Parenteral administration of estrogen should be avoided as it has been proven to be less effective than PGF [26] and also had negative effects on future reproductive performance [27].

The rationale for treatment of endometritis with PGF, or an analogue such as cloprostenol, is to stimulate uterine defense mechanisms by destroying the corpus luteum (CL) and removing the progesterone source [28, 29]. Some benefit may also be obtained by a PGF-induced transient increase in myometrial contraction promoting the expulsion of fluid and bacteria [28, 29], but increases in uterine contractility attributable to PGF therapy in the absence of a CL are thought to be negligible [30, 8]. Treatment of subclinical endometritis with cloprostenol between 20 and 33 in milk resulted in a 70% improvement in the risk of those cows becoming pregnant compared to their untreated counterparts [31]. Luteal status at the time of treatment was not reported, but it can be presumed, based on the results of other studies, that a large proportion of these animals had not yet had their first postpartum ovulation [32]. LeBlanc et al. [17] demonstrated that cows without palpable CLs treated for clinical endometritis between 20 and 26 days in milk had poorer pregnancy rates than their untreated counterparts, however; when treatment was delayed to between 27 and 33 days in milk there was an 18% improvement in pregnancy rate irrespective of luteal status. It was hypothesized that the use of PGF early in the postpartum period in those cows without a palpable CL caused premature lysis of luteal tissue that was needed for the establishment of normal estrous cycles. A similar study [29] did not report the same negative effect of PGF use. Perhaps this was because of much longer interval between treatment and first service. Based on these findings, it seems reasonable to recommend that PGF treatment be reserved for use in cows greater than 30 days in milk. Recently, it was demonstrated that the odds of pregnancy at the first service was the same when the Presynch-Ovsynch protocol was used on normal cows, and on those diagnosed with endometritis. Specifically, the protocol consisted of 2 injections of PGF 14 days apart starting at 35-42 days postpartum followed by the Ovsynch protocol (GnRH-PGF-GnRH) beginning 14 days after the second PGF injection [28]. Studies comparing the effectiveness of PGF to various other treatments have reported that PGF was at least equal to and perhaps better than the alternatives [8, 26], with the added benefit of milk residue avoidance and minimal contamination of the uterus [8]. The benefit of PGF
therapy may be nothing more than the occurrence of estrus which by nature is accompanied by a decline in progesterone concentrations. Cows without any detectable evidence of endometritis displaying estrus at least 3 times before the breeding season were reported to have improved fertility [33, 34]. Further studies directed at the physiologic benefits of PGF use in the postpartum period are warranted.

Intrauterine infusion of antimicrobials is aimed at obtaining greater concentrations of drug at the site of infection than would be achieved by systemic administration. Oxytetracycline was once the most popular agent infused in to the uterus of cows. Like most of the other intrauterine products it was poorly absorbed into the deeper layers of the uterus [26, 35]. Despite being largely limited to the uterine lumen meat and milk residues may also be a problem [36]; furthermore, both Lugol’s iodine and oxytetracycline cause coagulation necrosis of the endometrium [37]. Numerous other antibiotics and antiseptics have been used to treat endometritis. Several have been shown to be ineffective, or even harmful, for the treatment of uterine conditions while the performance of others is difficult to interpret because of lack of clear and consistent diagnostic criteria for uterine disease [18, 36]. Benzathine cephapirin (Metricure, Intervet Schering-Plough Animal Health) a first-generation cephalosporin with no meat or milk withdrawal requirement has largely replaced oxytetracycline as the drug of choice for uterine infusion in many countries including Canada. Most studies comparing intrauterine infusion of antimicrobials with PGF have generally failed to show any benefit to intrauterine infusion [8]; however, benzathine cephapirin is an exception. In a study involving 316 cows in 20 herds receiving cephapirin, PGF or no treatment for clinical endometritis is was shown that cows receiving cephapirin between 27 and 33 days in milk got pregnant 60% faster than the controls. Those receiving PGF got pregnant faster than the controls, but the difference was not significant [17]. Another field study involving 228 cows receiving cephapirin, PGF (cloprostenol) or no treatment between 20 and 33 days in milk showed that all cows, including those without evidence of subclinical endometritis (according to the cytological parameters used in that study), had a 62 to 63% increase in pregnancy rates compared to control animals. Cows diagnosed with subclinical endometritis showed respective 70 and 89% increases in pregnancy rates and significantly decreased median days open when treated with cephapirin and cloprostenol [18]. Metricure is available in Canada, but not in the United States. Intrauterine infusion with 125 mg of ceftiofur hydrochloride (Spectramast LC, Pfizer Animal Health), a broad-spectrum third-generation cephalosporin, to cows 44 days postpartum diagnosed with clinical endometritis reduced the prevalence of bacterial infections, but did not influence the proportion of animals with subclinical disease compared to untreated animals. Cows with subclinical endometritis experienced lower pregnancy rates compared to normal animals and treatment with ceftiofur early in the postpartum period did not influence the pregnancy rate. Interestingly, 69% of those animals diagnosed with subclinical endometritis did not have a concurrent bacterial infection which suggests that more study is required to determine the cost of aseptic endometritis and possibly to develop a different therapeutic approach [38].

More field studies are required to determine if individual herds should invest in the best possible diagnostic tests to identify every animal with endometritis (subclinical and clinical); and to determine if those herds should treat every case, or consider treating all postpartum cows on a systematic basis [8]. There is no doubt that these decisions will be influenced by the cost of diagnostic tests, the cost of treatment, the reproductive performance of the herd and the prevalence of disease [18]. Certainly there is evidence that over ½ of the cows with clinical endometritis had an otherwise normal postpartum period and would have gone untreated if only cows with an abnormal postpartum period (twins, retained placenta, metritis) had been selected for treatment [8]. Even if conservative values are assigned to the cost of a day open and multiplied by the average number of extra days a cow with endometritis is expected to require before she becomes pregnant it should be clear that endometritis is costing the dairy industry millions of dollars and is worthy of attention.
References


TECHNOLOGIES APPLIED TO EVALUATE FERTILITY OF BULL SEMEN: FRESH, FROZEN & SEXED

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Introduction:

In mammals, the sperm must transit in the female genital tract to undergo a series of biochemical modifications to be able to fertilize the mature oocyte. This process is known as capacitation and involves bicarbonate influx, activation of the cyclic AMP (cAMP) / protein kinase A pathway, tyrosine phosphorylation of various proteins, modification of membrane phospholipids, loss of cholesterol, hyperpolarization, intracellular alkalization and series of calcium influxes. These events lead to hyperactivated motility which is required to ascend the oviduct and culminates with the acrosome reaction in response to zona pellucida, an exocytotic event that allows for egg penetration (nicely reviewed in [1-5]).

Sperm quality and fertilizing potential are influenced by the integrity of the paternal genetic material and the integrity of the cell itself. Using current methods of semen evaluation, we can observe that different ejaculates can display the same level of sperm integrity but vary in terms of fertilization potential. Consequently, evaluation of semen cellular function must be conducted to a deeper level to properly address fertility. Semen from different bulls can vary from one another regarding their capacitation status. Moreover, sperm cells within the same ejaculate can also vary significantly as well.

To monitor in detail these biochemical variations, there is a need for sensitive tools to assess capacitation of spermatozoa and provide a better evaluation of the overall fertilizing potential of the ejaculate. Furthermore, these tools should be capable of single-cell analysis of each sample within a reasonable amount of time. Otherwise, the subtle differences are only overlooked and discrimination between semen with low or high fertility becomes difficult.

The analysis tool for fresh, frozen/thawed and sexed semen

Flow cytometry is a tool used in various fields of life science but its full potential is only beginning to be exploited in the field of reproductive biology. The principle is simple: a cell is loaded with a fluorescent molecule referred to as a “dye”. In order to produce fluorescence, the dye must be excited by a laser. Once a dye-loaded cell passes in front of the laser beam in the flow cytometer, the cell becomes fluorescent. Also present in the flow cytometer are photometers that allow cell detection and quantifies the fluorescence produced by the dye. Each dye can target a specific parameter (pH, as an example) and thus quantification of the fluorescence constitutes the measurement of that parameter.

Different dyes designed for various parameters related to DNA integrity or to capacitation status are available. The power of flow cytometry relies on the fact that it is able to

- analyze many dyes simultaneously,
- analyze thousands of cells in a single analysis, and
- perform this analysis within a few seconds.

As a result, flow cytometry can provide a global picture of the semen quality in a precise manner.

Computer assisted semen analysis (CASA) is another very useful tool and basically consists of a microscope linked to a computer that becomes the interface for image analysis. The software acquires sequential images from the semen sample loaded under the microscope and calculates sperm position on
each image. Then the positions are put into an algorithm that generates the sperm movement data. CASA allows the quantification of the percentage of cells with hyperactivated motility. In the bovine, hyperactivated motility in aqueous media is characterized by spermatozoa displaying high amplitude of lateral head displacement and low linearity of the path[6]. Such quantifications are impossible by simple subjective evaluation and even more problematic to standardize across artificial insemination (AI) centers.

DNA integrity

In the past, many tests like the TUNNEL assay or the Comet assay have been used to characterize DNA integrity in sperm cells [7]. The tool we use at L’Alliance Boviteq (LAB) is the SCSA test using acridin orange because of the consistency of the results when used with flow cytometry. Acridin orange fluorescence shifts from green to deep orange depending on its binding to double or single stranded DNA respectively. In each cell measured, the percentage of orange fluorescence is determined and constitutes an evaluation of the integrity of the paternal genetic material. In our setting for instance, bulls with more than 10% of their sperm in the high orange fluorescence window display significantly lower fertility.

Sperm integrity

Many events triggering capacitation depend upon cytoplasmic membrane integrity. Thus dyes like SYBR, Hoechst and propidium iodide that evaluate cell viability are convenient dyes to assess general semen quality. Additionally, the integrity of cellular organelles is important to monitor. For instance, acrosomal integrity must be intact for sperm to penetrate oocytes. This parameter can be monitored with flow cytometry by using labelled agglutinins as dyes. Another important parameter to monitor is mitochondrial potential. In order to produce ATP, mitochondria must maintain an electric potential across their membranes. Many dyes like JC1 and mitotracker dyes can be used on sperm cells to measure the integrity of the mitochondria. These steps were nicely reviewed by Barend Gadella and his co-workers [8].

These parameters are presently monitored at LAB to assess semen quality. However, these parameters alone do not provide a complete prediction of the observed semen fertility following AI. As an example two bulls with very similar global sperm integrity can yield very different fertilities. Their sperm cells must differ on more subtle, yet very important, physiological aspects.

Figure 1: Analysis of frozen thawed bovine sperm using four dyes with a BD LSRII flow cytometer. A. Hoechst 33342 dye to select sperm (right peak) from the debris (left peak). B. PNA-FITC dye to assess acrosomal integrity (right peak = acrosomal damage). C. PI dye for membrane integrity (right peak = membrane damage). D. Mitotracker dye for mitochondrial function (right peak = sperm with intact mitochondria).
Studying capacitation

Capacitation is a dynamic process that is necessary for spermatozoa to acquire their fertilizing potential. Research teams around the world are continuously conducting research studies to characterize this process giving reproductive biologists new niches to develop innovative diagnostic tools that can be applied to bovine semen.

Early in the process of capacitation, bicarbonate enters the sperm cell most likely via a sodium/bicarbonate co-transporter [9]. This transient rise in bicarbonate activates specific enzymes [10] thereby leading to changes in the distribution of lipids of the plasma membrane. These changes facilitate cholesterol efflux from the membrane toward bovine serum albumin and would prepare sperm for fertilization [11]. Logically, monitoring lipid dynamics can provide useful information.

The cytoplasm of spermatozoa is negatively charged compared to the exterior of the cell. Before capacitation, the potential is -30mV. During capacitation, the influx of bicarbonate increases the intracellular pH and also causes a rise in cAMP by soluble adenylate cyclase activation [12]. Alkalization and increased cAMP content are believed to induce the opening or closing of different ionic channels which causes the sperm cell to hyperpolarize to -60mV [13-16]. Characterizing these events in more details with flow cytometry could reveal interesting information.

Hyperpolarization and calcium influx are required to achieve acrosome reaction [1]. Hyperpolarization would be required to activate voltage-dependent calcium channels following the interaction with the zona pellucida. A transient calcium influx in sperm [17, 18] is thought to trigger the inositol trisphosphate (IP3) pathway resulting in a sustained calcium influx [19-21]. Other pathways of calcium rise involving CatSper channels have also recently been reported [22]. Calcium currents and calcium signalling are key events in capacitation and warrant further characterization to fully understand sperm function.

What to do with the data from the flow cytometer and the CASA?

At present, research efforts are concentrated at building reference values with proper controls for each parameter. For every semen analysis, spermatozoa parameters including ion concentrations, intracellular pH, membrane integrity and motility are acquired from a control freeze lot. If the values obtained are within an acceptable range from the lot’s own historical average, the analysis is considered valid (internal control). Then, data sets are acquired from frozen/thawed lots of known fertility. Over a hundred lots are analyzed and plotted on a frequency histogram. This reference database is becoming the multi-parametric tool to which we compare any semen (see Figure 2). We use this tool to select bulls, control semen production quality and to identify specific therapies for bulls of high genetic potential that we want to rescue.
**Figure 2:** Illustration of a hypothetical reference value graph. Data ranges from 46 to 63 arbitrary units with 5% of the observations considered as outliers (red bars).

**What’s next?**

Research constantly yields a better understanding of male reproductive functions and new ideas to predict fertility. Particularly, proteomic is a very powerful tool that constantly brings new interesting candidates to our sight. Flow cytometry is a wonderful tool to study proteins since once candidates have been identified by proteomic, they can be quantified in sperm cells using fluorescent antibodies as dyes. Specific protein quantification by flow cytometry is under development at LAB.

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FIELD RESULTS USING SEXED SEMEN: AN ITALIAN EXPERIENCE

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Introduction

One of the most significant advantages to embryo transfer work is the resulting female progeny from genetically superior donors. Since 2001, we have offered our clients the service of sexing embryos using the YCD system. However, only 10% of embryo collections involve the sexing of embryos. This is mainly explained by (i) the high cost of the technique; and (ii) the need for a qualified technician in order to save time on this lengthy procedure.

In Italy, sexed semen has been made available by several AI centers since late 2007. This presents a great opportunity to produce female embryos at a lower cost and in less time. The aim of this field trial is to compare superovulation efficiency between donors inseminated with sexed semen (sorted by flow cytometry) and those inseminated with conventional frozen semen.

Superstimulation protocol

All donors were examined from seven to nine days after their heat in the cycle. At the beginning, follicle dynamics were controlled using Cidr/Prid + 2.5 mg of EB + FSH four days later or by dominant follicle ablation via intravaginal puncture + FSH one or two days later. However, since the latter technique tends to produce more viable embryos, we are now carrying out superstimulation programs from day 9 to 12 in the cycle in relation to scheduled appointments. Cidr/prid are used only when there are several donors to program that are not all between 9 and 12 d of the cycle. We use the same superstimulation protocol for sexed or unsexed semen, which consists of 9 decreasing dose injections at 12 hrs apart as follows: for early lactating cows, a total dose of 900-1000 UI of Pluset; for the late ones, a total dose of 750 UI, for dry cows, a total dose of 500-600 UI, and for virgin heifers, a total dose of 350UI. A double prostaglandin dose injections are administrated at Day 4 along with the seventh and eighth FSH treatment. Heat usually occurs at Day 6 and embryo collection is carried out seven days after heat.

Artificial insemination

Breeders usually inseminate donors through the uterine body. In general, when using the unsexed semen each donor is inseminated 14 hrs after estrus detection and again after 8 hrs interval. When using sexed semen (approx 2 million sperm) the first insemination is performed at least 16-17 hrs after heat detection. AI is repeated every 4-5 hrs up to three times. This practice aims to maximize sperm availability during ovulation.

Recipient synchronization

Recipients are synchronized with one dose of PGF2α 24 hrs before the donor gets the PGF2α. In general, recipients are not examined before the PGF2α injection. The breeder must record heat signs (standing heat) and blood discharge. In certain situations a Cidr is used for seven days followed by a prostaglandin injection, the goal being to improve the number of available recipients on a given day. Generally speaking, when recipients are well managed breeders tend to have good results in AI and we have good results in ET as well.

Experimental design

Data from 150 embryo collections made from conventional semen, and 60 collections made with sexed semen have been analysed by the analysis of variance (GLM-ANOVA) using Systat 11.0. Some
parameters have been analysed as follows: parity, embryo grade, number of transferable embryos (TE), degenerated embryos (DEG), number of unfertilized oocytes (UFO), number of semen doses used, and the various AI centers.

Results
Donors were randomly inseminated with sexed semen whenever possible. In many situations, breeders were deciding themselves whether or not to use sexed semen. The number of transferable (TE) embryos (grade 1+2+3) was higher with conventional semen than with sexed semen (5.84 vs 3.76; P≤0.011). The number of embryos qualifying for freezing (grade 1+2) was higher with conventional semen than with sexed semen (5.52 vs 3.64; P≤0.019). The number of unfertilised oocytes (UFO) was significantly higher with sexed semen (5.24 vs 2.67; P≤0.000) whereas no difference was found in the number of degenerated embryos (DEG) (0.99 vs 1.02) produced by both types of semen.

The amount of inseminations seems to have some impact on the number of good embryos recovered. We used 2 (n= 1), 3 (n=38), 4 (n= 18) and 6 (n= 3) doses of sexed semen. We experienced better results using 4 doses (TE: 1.00 vs 3.18 vs 5.16 vs 3.50; P> 0.05, DEG: 1.00 vs 0.97 vs 1.06 vs 1.50 (P>0.05) and UFO:2.00 vs 4.87 vs 5.89 vs 8.00; P>0.05);

We used semen from four different AI centers; however, only two of these AI centers could enter in our analysis. There was no difference between the two AI centers in the number of either TE (4.36 vs 3.80; P> 0.05), DEG: 1.44 vs 0.26 P>0.05 or UFO (4.31 vs 6.07; P> 0.05).

To test the effect of parity using sexed semen, we divided donors into three groups: 1) heifers and primiparae (n= 8), 2) second and third lactation cows (n= 27) and 3) four lactation ones and over (n= 25). The number of TE was 2.37 vs 5.22 vs 2.58 (P<0.05, DEG_0.88 vs 1.30 vs 0.75 and UFO was 3.62 vs 5.15 and 5.87 (P<0.05) between the three groups, respectively. Of the same 3 groups using conventional semen, the number of TE was 8.41 vs 5.94 and 4.45 (P<0.017), DEG_1.20 vs 1.07 vs 0.75 and UFO 0.92 vs 2.37 and 4.00(P<0.015). In group 1, due to the limited amount of collections the difference between sexed and non sexed semen is not significant. In group 2, we got 44.7% of TE with sexed semen vs 62.4% with conventional semen (P=0.026). In group 3, we got 28.6% of TE with sexed semen vs 48.3% with conventional semen. It should be noted that in our particular situation, the low number of collections using sexed semen in group 1 was mainly due to a higher demand for males by AI centers, therefore conventional semen was the obvious choice.

Discussion
Our data indicate that when using sexed semen we were recovering less transferable embryos (an average of two per collection in our case). Interestingly though, even if the number of TE’s recovered were smaller, using sexed semen would yield more female embryos (almost 1 more female per collection in our data). It is noteworthy to mention that only two male pregnancies have been reported from sexed semen which represents approximately 1% of all pregnancies obtained from sexed semen. Other benefits include a need for less recipients, and a reduced need to freeze embryos. This results in a reduction of veterinary labour involved, therefore adding a cost advantage despite the higher price of sexed semen. These are the main reasons why breeders consider using sexed semen for ET, despite being conscious of the risk of an increased number of unfertilized ova.

Our goal for the future is to broaden the practice of splitting female embryos. We also need AI centers to diversify their bank of sexed semen so as to include a better variety of superior genetic bulls, as well as providing more information concerning the fertility of the sexed semen. In fact, we believe that using doses with a higher concentration of sexed sperm is not as efficient as obtaining sexed sperm from bulls with a high fertility rate.

1 *Bioniche Animal Health (Europe) Ltd.*
SUPEROVULATION PROGRAMS IN CATTLE

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Introduction

Although research efforts in recent years have resulted in no increase in the number of transferable embryos per treatment, protocols that control emergence of the follicular wave (Bo et al. 1995; 2002a) and ovulation (Baruselli et al., 2006; Bó et al., 2006) have allowed the superstimulation of groups of donors, regardless of the stage of the estrous cycle and permitted fixed-time artificial insemination (FTAI) of donors, without the need to detect estrus. This has had a positive impact on commercial embryo transfer, because it has facilitated the scheduling of working protocols, without being dependent on the knowledge and skill of personnel to detect estrus. However, the complexity of protocols can lead to errors that will result in a decrease in superovulatory response, or even a complete lack of response. In this regard, the need to inject FSH every 12 h is a factor of greatest concern (Bo et al., 1994). Moreover, the most commonly used treatment for synchronization of follicular wave emergence for superovulation involves the use of a progesterone releasing device and estradiol (Mapletoft et al., 2002), which cannot be used in many countries because of concerns about the effects of estrogens in the food chain (Lane et al., 2008). The objective of this paper is to present progress in the development of alternative treatments that do not require the use of estradiol to synchronize follicular development, the simplification of superstimulation protocols by the administration of a single injection of FSH and the use of eCG at the end of superstimulation treatments to increase the number of transferable embryos obtained per embryo collection.

Manipulation of the follicular wave for superovulation

Traditionally, superstimulation treatments were initiated during the mid-luteal phase, approximately 9 to 11 days after estrus (Lindsell et al. 1986). This is because the second follicular wave usually emerges 9 or 10 days after estrus (Ginther et al., 1989). However, a greater superovulatory response occurred when treatments were initiated on the day of follicular wave emergence rather than 1 or 2 days later (Nasser et al., 1993, Adams et al., 1994). Therefore, conventional treatment protocols have two drawbacks: 1) the requirement to have trained personnel dedicated to the detection of estrus, and 2) the necessity to have all donors in estrus at the same time in order to begin the superstimulatory treatments at the beginning of a follicular wave.

In the 90’s, we reported on the use of progestins and estradiol esters to induce follicular atresia and synchronous emergence of a new follicular wave, on average, 4 days later (Bo et al., 1995). This treatment has been used by practitioners around the world (Mapletoft et al., 2002; Bo et al., 2006; Baruselli et al., 2006), but its use has been restricted recently in countries like USA, New Zealand and the European Union. This restriction leaves many embryo transfer practitioners with a serious dilemma and created the need to develop treatments that do not involve the use of estradiol esters.

An alternative to estradiol is to eliminate the suppressive effect of the dominant follicle by ultrasound-guided follicle aspiration and initiate superstimulatory treatments one or two days later (Bungarts and Niemann, 1994; Bergfelt et al., 1997). The disadvantage is that this approach requires ultrasound equipment and trained personnel which is only appropriate for embryo production centers, where all the donors are concentrated in the same location; it is very difficult to apply in the field.
Another approach to the synchronization of follicular wave emergence is to use pLH or GnRH to induce ovulation of the dominant follicle (Macmillan and Thatcher, 1991) which is followed by wave emergence 1.5 to 2 days later (Pursley et al., 1995). However, this occurs by chance (stage of the cycle) or only when the treatment results in ovulation (Martinez et al., 1999). Pursley et al. (1995) reported that the administration of GnRH at random stages of the cycle induced ovulation in about 85% of dairy cows, but more recently, Colazo et al. (2007) reported an ovulation rate of 62.4% in lactating dairy cows receiving 25 mg pLH (Lutropin-V, Bioniche Animal Health, Belleville, ON, Canada) and 44.3% in those receiving 100 µg GnRH. In another study in beef heifers, pLH induced ovulation in 78% of beef heifers and following GnRH ovulation occurred in 56%, (Martinez et al., 1999). The occurrence of ovulation following administration of GnRH or pLH in beef cows appears to be similar to heifers (<60%; Small et al., 2009). Not surprisingly, treatment with GnRH at random stages of the cycle prior to initiating superstimulatory treatments resulted in lower responses than treatments initiated after follicular aspiration or estradiol (Deyo et al., 2001). However, in a retrospective analysis of commercial suprovulations, Hinshaw found no differences in the number of embryos transferred between donors superstimulated 4 days after treatment with estradiol-17β and progesterone (7.8 transferable embryos, n = 1136) and those superstimulated 2 days after treatment with GnRH (7.7 transferable embryos, n=56; personal communication; AETA 2007). In another recent study (Wock et al., 2008), CIDR-treated dairy cows (n = 411) were superstimulated 4 days after the administration of estradiol or 2 days after GnRH to synchronize follicular wave emergence. Data analysis revealed no significant differences in the number of ova/ embryos or transferable embryos between groups. In another retrospective analysis of commercial data, dairy donors (n = 245) superstimulated 60 h after the administration of GnRH produced similar numbers of transferable embryos as those (n = 691) superstimulated 4 days after the administration of estradiol-17β. Although these commercial data appear very promising, properly controlled studies with the use of GnRH must be done to confirm these results.

Superstimulation during the first follicular wave

Follicle wave emergence occurs consistently at the time of ovulation (Ginther et al., 1989), and superstimulation treatments initiated at the time of emergence of the first follicular wave in cows (Nasser et al. 1993) and sheep (Menchaca et al., 2002) have resulted in satisfactory results. Adams et al. (1994) also reported no difference in superovulatory response when FSH treatments were initiated at the time of emergence of the first or second follicular wave. However, success relies upon successful determination of the time of ovulation or accurate estrus detection with ovulation expected to occur 1 day after the onset of estrus.

To avoid the need to observe the expression of estrus in Nelore (Bos indicus) donors, Nasser et al. (2003) induced a synchronous ovulation with an estradiol/CIDR protocol followed by pLH 24 h after CIDR withdrawal. Superstimulation treatments were initiated 24 h after pLH (i.e. the expected time of ovulation and emergence of the first follicular wave). Superovulatory response and the number of transferable embryos did not differ from a contemporary group superstimulated 4 days after treatment with estradiol. However, the number of transferable embryos was reduced in cows superstimulated during the first follicular wave without an accompanying use of a CIDR. These results suggest that circulating progesterone is required for oocyte maturation, and fertilization in superovulated donors.

Recently, we conducted a series of five experiments with the overall objective of developing a protocol for superstimulation during the first follicular wave, using progesterone releasing devices but not estradiol. We considered previous reports showing that ovulatory response to GnRH could be increased by the administration of PGF30 to regress the CL at the time of insertion a progesterone releasing device that was left in place for 7 to 10 d. Administration of GnRH at the time of device removal resulted in ovulation and follicular wave emergence 1 to 2 days later (Small et al., 2009).

In the first experiment (Carballo et al., 2008a), 70 Bonsmara donors (29 cows and 41 heifers) were randomly assigned to one of two treatment groups. Donors in the First Wave Group received a
progesterone releasing device (1.56 g of progesterone, Cue-Mate, Bioniche Animal Health) along with a dose of PGF$_{2\alpha}$ (0.150 mg D (+) cloprostenol, Bioprost-D, Biotay SA, Argentina) at random stages of the estrous cycle. Cue-Mates were removed 10 days later and a second PGF$_{2\alpha}$ was administered at that time, followed by GnRH (0.050 mg Lecirelina, Biosis-OV, Biotay SA) 36 h later. Ovulation was expected to occur within 36 h after GnRH. On Day 0 (36 h after GnRH), donors received a new Cue-Mate and treatment with a total dose of 200 to 260 mg (heifers) or 320 mg (cows) NIH-FSH-P1 of Folltropin-V (Bioniche Animal Health) in twice daily decreasing doses over 5 days was initiated. PGF$_{2\alpha}$ was administered with the last two Folltropin-V treatments and Cue-Mate devices were removed with the last Folltropin-V. All donors received 12.5 mg pLH 24 h after Cue-Mate removal and were FTAI 12 and 24 h later. Ova/embryos were collected 7 days after pLH. Donors in the Control Group received a Cue-Mate and 2 mg EB (Bioestradiol, Biotay SA) and 50 mg of progesterone (Progesterona Rio de Janeiro, Laboratorios Allignani Hnos SRL, Argentina) and Folltropin-V treatments were initiated 4 days later with the same dosages and treatment protocol as the First Wave Group. There were no significant effects of treatment or donor category (cows vs heifers) on superovulatory response and embryo quality. The numbers of transferable embryos were 5.1 ± 0.9 and 3.7 ± 0.8 for donors in the Control and First Wave groups, respectively.

Although this study showed that superstimulation during the first follicular wave was as efficacious as the “standard” estradiol treatment protocol in beef cattle, the duration of the protocol (26 days vs 15 days from Cue-Mate insertion until embryo collection) made it time-consuming and difficult to implement. Therefore, a series of studies were designed to shorten and simplify the protocol. In the first study (Carballo et al., 2008b), superovulatory response was compared between donors that were pretreated with a Cue-Mate for 10 days vs those pretreated with a Cue-Mate for 5 days. Ovulation rate and the interval from GnRH administration to ovulation was 81.8% (9/11) and 32.0 ± 2.8 h vs 100% (11/11) and 36.0 ± 0.0 h for cows pretreated with Cue-Mate devices for 10 or 5 days, respectively (P>0.9). Furthermore, the number to transferable embryos did not differ (P>0.9) between groups (Table 1).

Table 1. Superovulatory response (means ± SEM) in Brangus cows and heifers treated with Folltropin-V during the first follicular wave synchronized by a 10-day or 5-day pretreatment with a Cue-Mate device as compared to 4 days after administration of 2.5 mg EB, 50 mg progesterone and insertion of a Cue-Mate device.

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<th>Groups</th>
<th>n</th>
<th>Total ova/embryos</th>
<th>Fertilized ova</th>
<th>Grade 1 &amp; 2 embryos</th>
<th>Grade 1,2 &amp; 3 embryos</th>
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</thead>
<tbody>
<tr>
<td>Cue-Mate 10 days</td>
<td>11</td>
<td>11.3 ± 2.9</td>
<td>7.2 ± 2.2</td>
<td>4.8 ± 1.4</td>
<td>5.5 ± 1.6</td>
</tr>
<tr>
<td>Cue-Mate 5 days</td>
<td>11</td>
<td>9.8 ± 3.0</td>
<td>7.4 ± 2.7</td>
<td>4.9 ± 2.3</td>
<td>5.2 ± 2.6</td>
</tr>
<tr>
<td>Control (EB+P4)</td>
<td>11</td>
<td>9.6 ± 2.7</td>
<td>6.0 ± 1.5</td>
<td>4.1 ± 1.5</td>
<td>5.4 ± 1.6</td>
</tr>
<tr>
<td>P Value</td>
<td>0.9868</td>
<td>0.9631</td>
<td>0.9873</td>
<td>0.9472</td>
<td></td>
</tr>
</tbody>
</table>

A third experiment (Carballo et al., 2009) was designed to determine whether it was necessary to remove the progesterone releasing device during the superstimulation protocol. Angus cows (n=27) and heifers (n=10) were superstimulated by two treatments in a cross over design. All donors received a Cue-Mate along with PGF$_{2\alpha}$ at random stages of the estrous cycle; those in Group 1 received a second PGF$_{2\alpha}$ at the time of Cue-Mate removal 5 days later, followed by GnRH 36 h later. On Day 0 (36 h after GnRH) donors received a new Cue-Mate and superstimulation treatments were initiated with a total dose of 400 mg NIH-FSH-P1 of Folltropin-V in twice daily decreasing doses over 5 days. PGF$_{2\alpha}$ administration, Cue-Mate
removal, pLH administration, FTAI and embryo collections were done as in previous studies. Donors in Group 2 were treated similarly to those in Group 1, except that Cue-mate devices were not removed but remained in place for 13 days (i.e., were removed with the last FSH and PGF<sub>2α</sub> injection). Ovulation rate and the interval from GnRH treatment to ovulation was 86.5% (64/74) and 35.6±1.6 h for donors in which the Cue-Mate was replaced during the treatment (Group 1) and 89.2% (33/37) and 37.5 ± 0.7 for those in which the Cue-Mate was not replaced until the last FSH and PGF<sub>2α</sub> injection. Mean (± SEM) numbers of ova/embryos and transferable embryos were not different between groups (Table 2), indicating that it was not necessary to remove the progestin device to synchronize ovulation (and follicle wave emergence for superstimulation) with GnRH.

Table 2. Superovulatory response (means ± SEM) in Angus cows and heifers treated with Folltropin-V during the first follicular wave synchronized by insertion of a Cue-Mate device followed 7 days later with the administration of GnRH.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Total ova/embryos</th>
<th>Fertilized ova</th>
<th>Grade 1 &amp; 2 embryos</th>
<th>Grade 1,2 &amp; 3 embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1*</td>
<td>37</td>
<td>8.2 ± 1.0</td>
<td>4.8 ± 0.7</td>
<td>3.8 ± 0.6</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>Group 2**</td>
<td>37</td>
<td>9.8 ± 0.9</td>
<td>6.8 ± 0.8</td>
<td>5.3 ± 0.7</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>P Value</td>
<td></td>
<td>0.1964</td>
<td>0.1413</td>
<td>0.2720</td>
<td>0.1970</td>
</tr>
</tbody>
</table>

* Cue-Mate devices were removed 5 days after insertion and replaced with a new Cue-Mate on the day that Folltropin-V treatments were initiated.

** Cue-Mate devices were not replaced during the whole superstimulation protocol.

A fourth study (Carballo et al., 2009) was designed to evaluate the effect of giving Folltropin-V for 4 days vs 5 days. Simmental (n=18) and Angus (n=6) cows were superstimulated by the two treatment protocols in a cross over design. Cows in both groups were treated similarly to those in Group 2 in the previous experiment (i.e., Cue-Mates were not replaced during treatment). Cows in Group 1 (control) received FSH over 5 days; while those in Group 2 received the same dosage of Folltropin-V, but given in twice daily decreasing doses over 4 days (Cue-Mates were removed with the last FSH and PGF<sub>2α</sub> injections). Mean (± SEM) numbers of ova/embryos and transferable embryos were not different between groups (P>0.6; Table 3).

Table 3. Superovulatory response (means ± SEM) in Simmental and Angus cows superstimulated during the first follicular with Folltropin-V, given twice-daily for 4 or 5 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Total ova/embryos</th>
<th>Fertilized ova</th>
<th>Grade 1 &amp; 2 embryos</th>
<th>Grade 1,2 &amp; 3 embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>24</td>
<td>13.5±2.4</td>
<td>8.2±1.4</td>
<td>6.2±1.1</td>
<td>6.6±1.1</td>
</tr>
<tr>
<td>Group 2</td>
<td>24</td>
<td>12.0±1.9</td>
<td>7.0±1.2</td>
<td>5.0±0.9</td>
<td>5.8±1.0</td>
</tr>
<tr>
<td>P Value</td>
<td></td>
<td>0.7543</td>
<td>0.5638</td>
<td>0.4934</td>
<td>0.6542</td>
</tr>
</tbody>
</table>

A final experiment was designed to determine whether it was possible to further simplify the first wave protocol by reducing the number of PGF<sub>2α</sub> treatments during the pre-treatment. A second objective was to confirm the effectiveness of the first wave protocol by comparing it with the protocol in which
progesterone and estradiol was used to synchronize follicle wave emergence. Simmental donors cows (n=14) were assigned randomly to three treatment groups in a cross-over design so that all animals received all treatments. Donors in Groups 1 and 2 received Cue-Mates as in the previous experiments, but those in Group 1 received PGF$_{2\alpha}$ at the time of Cue-Mate insertion and 5 days later (as in previous experiments), whereas those in Group 2 received PGF$_{2\alpha}$ at Cue-Mate insertion only (i.e., eliminating the need to handle animals on Day 5). Cows in Groups 1 and 2 received GnRH 7 days after Cue-Mate insertion to induce ovulation and treatments with Folltropin-V were initiated 36 h later (Day 0; as in previous experiments). Donors in Group 3 (EB + P4) received a Cue-Mate plus 2.5 mg estradiol benzoate and 50 mg of progesterone at random stages of the estrous cycle and superstimulation treatments were initiated 4 d later. All cows received 400 mg of Folltropin-V in twice daily injections over 4 days as described in the previous experiment. Mean numbers of ova/embryos, fertilized ova and transferable embryos did not differ among groups (Table 4).

In conclusion, data demonstrate that superstimulation during the first follicular wave (synchronized by insertion of a Cue-Mate device and GnRH administration 7 days later) or 4 days after administration of 2.5 mg EB, 50 mg progesterone and insertion of a Cue-Mate device.

### Table 4. Superovulatory response (means ± SEM) in Simmental cows treated with Folltropin-V during the first follicular wave (synchronized by insertion of a Cue-Mate device and GnRH administration 7 days later) or 4 days after administration of 2.5 mg EB, 50 mg progesterone and insertion of a Cue-Mate device.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Total ova/embryos</th>
<th>Fertilized ova</th>
<th>Grade 1 &amp; 2 embryos</th>
<th>Grade 1,2 &amp; 3 embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1* (2 PGF$_{2\alpha}$)</td>
<td>14</td>
<td>12.9±2.0</td>
<td>9.8±1.7</td>
<td>5.9±1.2</td>
<td>6.6±1.2</td>
</tr>
<tr>
<td>Group 2** (1 PGF$_{2\alpha}$)</td>
<td>14</td>
<td>11.5±1.7</td>
<td>9.3±1.5</td>
<td>7.2±1.5</td>
<td>7.7±1.6</td>
</tr>
<tr>
<td>Group 3 (EB + P4)</td>
<td>14</td>
<td>14.5±2.8</td>
<td>9.4±2.3</td>
<td>5.6±1.5</td>
<td>6.8±1.7</td>
</tr>
<tr>
<td>P Value</td>
<td>0.8033</td>
<td>0.9220</td>
<td>0.5416</td>
<td>0.8547</td>
<td></td>
</tr>
</tbody>
</table>

*Group 1: Donors received PGF$_{2\alpha}$ at Cue-Mate insertion and 5 days later.  
** Group 2: Donors received PGF$_{2\alpha}$ at Cue-Mate insertion only.

In conclusion, data demonstrate that superstimulation during the first follicular wave can be used successfully in groups of randomly cycling donors without the need for estrus detection or estradiol to synchronize follicular wave emergence. The protocol is easy to follow and embryo production is comparable to that of the estradiol and progesterone protocol. The recommended protocols are the following:

**Protocol 1. Folltropin-V given in twice daily injections over 4 days**

- **Day -8 AM (Tuesday):** insert a Cue-Mate and inject PGF$_{2\alpha}$ im
- **Day -1 AM (Tuesday):** inject GnRH or Lutropin-V im
- **Day 0 PM (Wednesday):** (36 h after GnRH) begin superstimulation with Folltropin-V
- **Day 3 PM (Saturday):** inject PGF$_{2\alpha}$ im
- **Day 4 AM (Sunday):** remove Cue-Mate and inject PGF$_{2\alpha}$ im and last FSH
- **Day 5 AM (Monday):** inject GnRH or Lutropin-V im
- **Day 5 PM (Monday):** first AI
- **Day 6 AM (Tuesday):** second AI
- **Day 13 AM (Monday):** embryo collection
Protocol 2. Folltropin-V given in twice daily injections over 5 days

Day -8 AM (Monday): insert a Cue-Mate and inject PGF$_{2_{a}}$ im
Day -1 AM (Monday): inject GnRH or Lutropin-V im
Day 0 PM (Tuesday): (36 h after GnRH) begin superstimulation with Folltropin-V
Day 4 PM (Saturday): inject PGF$_{2_{a}}$ im
Day 5 AM (Sunday): remove Cue-Mate and inject PGF$_{2_{a}}$ im and last FSH
Day 6 AM (Monday): inject GnRH or Lutropin-V im
Day 6 PM (Monday): first AI
Day 7 AM (Tuesday): second AI
Day 14 AM (Monday): embryo collection

Superstimulation using a single administration of FSH

Traditional superstimulatory treatments consisted of a single administration of equine chorionic gonadotrophin (eCG) or twice daily injections of pituitary extracts containing FSH over 4 or 5 days (Mapletoft et al., 2002). The eCG is a complex glycoprotein which has a long half-life (over 40 h) which represents a practical advantage, since a single administration will induce ovarian superstimulation (Schams et al. 1977, Murphy and Martinuk, 1991). However, prolonged stimulation with eCG causes an increased number of unovulated follicles at the time of embryo collection which affects collection efficiency and embryo quality (González et al., 1994). In contrast, the half-life of FSH is 5 h in the cow (Laster, 1972; Demoustier et al., 1988) and requires frequent applications to induce superstimulation (Bellows et al. 1969; Monniaux et al., 1983). Twice daily treatments with FSH have resulted in greater superovulatory response than once daily administration (Looney et al. 1981; Monniaux et al., 1983, Walsh et al., 1993).

The need to inject FSH twice a day requires constant attention by farm-personnel and increases the possibility of failures due to mishandling and errors in giving the treatments. In addition, twice daily treatments can cause undue stress in donor cows with a subsequent decreased superovulatory response (Edwards et al., 1987; Bo et al., 1994), and/or altered preovulatory LH surge (Stoebel and Moberg, 1982). Therefore, simplified protocols of superstimulation may be expected to reduce donor handling costs and improve response, particularly in less tractable animals.

More than 10 years ago, we reported that a single subcutaneous administration of 400 mg NIH-FSH-P1 of Folltropin-V in beef cows with good body condition (> 3 out of 5), resulted in a superovulatory response equivalent to the traditional treatment protocol of twice daily injections over 4 days (Bo et al., 1994). However, the results could not be repeated in Holstein cows, which had less subcutaneous fat (Hockley et al., 1992). In a subsequent study in Holstein cows, the single injection was split into two, with 75% of the dose of Folltropin-V administered subcutaneously on the first day of treatment and the remaining 25% administered 48 h later, when PGF$_{2_{a}}$ is normally given (Lovie et al., 1994). Superovulatory response was intermediate, between that obtained with the traditional protocol (the highest response) and that obtained with a single subcutaneous injection (the lowest response).

An alternative to induce a consistent superovulatory response with a single injection of FSH would be to combine the pituitary extract with agents that result in a slow and steady release of the hormone for several days. These agents are commonly known as polymers, are biodegradable and non-reactive in the tissue, facilitating use in animals (Sutherland, 1991). Yamamoto et al. (1994) reported that FSH combined with a 30% solution of polyvinylpyrrolidone (PVP) and administered in a single intramuscular injection resulted a comparable response to the traditional treatment of multiple injections over 4 days. However, Callejas et al. (2002) and Bo and Mapletoft (unpublished observations) were unable to induce a satisfactory superovulatory response with this compound. However, FSH dissolved in PVP and combined with eCG resulted in a superovulatory response that was similar to the traditional twice-daily FSH treatment (Callejas et al., 2002). Kimura et al. (2007) reported that a single application of FSH in
aluminum hydroxide gel was effective in inducing a superovulatory response in cattle and Choi et al. (2002) showed that FSH dissolved in polyethylene glycol (PEG), resulted in a superovulatory response similar to that obtained by treatment with twice-daily FSH injections over 4 days. However, aluminum hydroxide is commonly used as a vaccine adjuvant (Baylor et al., 2002) which may preclude its use for superstimulation.

We have recently conducted a series of experiments to determine the superovulatory response of donors treated with a single injection of Folltropin-V diluted in a slow release formulation (SRF, Bioniche Animal Health). On Day 0 (beginning of treatment), all cows received 5 mg estradiol-17β plus 50 mg progesterone and a Cue-Mate device and on Day 4 treatments with Folltropin-V were initiated. Cows either received twice-daily intramuscular injections of Folltropin-V over 4 days or a single intramuscular injection in the neck. The single injection was prepared by diluting the Folltropin-V lyophilized powder in 1 mL of saline for injection and mixed with 9 mL of the SRF in the syringe, immediately before administration. In the AM and PM of Day 6, all cows received PGF₂α and Cue-Mates were removed in the PM. In the AM of Day 8, cows received 12.5 mg pLH and were FTAI 12 and 24 h later. Ova/embryos were collected non-surgically on Day 15 and evaluated. Summarized results from 5 experiments involving 325 embryo collections in Angus, Brangus, Braford and Bonsmara donors are shown in Table 5. Mean numbers of ova/embryos, fertilized ova and transferable embryos did not differ between groups (Table 5). These results suggest that it is possible induce a consistent superovulatory response following administration of a single injection of Folltropin-V, without adversely affecting the number of transferable embryos.

Table 5. Mean (± SEM) ova/embryo production in Angus, Brangus, Braford and Bonsmara donors treated with Folltropin-V given by twice daily intramuscular injections over 4 days (Control) or diluted in SRF and given by a single intramuscular injection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Total ova/embryos</th>
<th>Fertilized ova</th>
<th>Grade 1 &amp; 2 embryos</th>
<th>Grade 1,2 &amp; 3 embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>161</td>
<td>12.2±0.6</td>
<td>8.5±0.5</td>
<td>5.7±0.3</td>
<td>6.5±0.4</td>
</tr>
<tr>
<td>Single Injection</td>
<td>164</td>
<td>12.0±0.8</td>
<td>8.3±0.6</td>
<td>5.8±0.4</td>
<td>6.4±0.4</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.3584</td>
<td>0.2746</td>
<td>0.5617</td>
<td>0.3322</td>
</tr>
</tbody>
</table>

Use of eCG in the final stages of the superstimulatory treatment

Speculations have been made for some time about the need for FSH and LH during superstimulatory treatments. Basic studies on follicular development have shown that FSH is required for follicle recruitment and growth, until the dominant follicle reaches 8.5 mm in diameter in Bos taurus cows (Ginther et al., 1996) and 6.2 mm in Bos indicus cows (Gimenes et al. 2005, Sartorelli et al., 2005). After that size, the dominant follicle acquires LH receptors on granulosa cells and becomes LH dependent (reviewed in Evans and Mihm, 2008). Therefore, follicles of superstimulated cows could benefit from the inclusion of a greater amount of LH at the end of treatment. Price et al. (1999) tried to increase LH pulse frequency by frequent applications of GnRH. Despite achieving an increase in estradiol production by the growing follicles, only one donor ovulated after an injection of GnRH, probably due to desensitization the pituitary gland to GnRH. Another option might be to use eCG at the end of a conventional treatment with FSH. eCG is a gonadotropin with FSH and LH activity (Steward et al. 1976; Litch et al. 1979; and Martinuk Murphy, 1991) that has a long half-life (40 h). Therefore, eCG could provide a constant stimulus to the LH receptors of the growing follicles at the end of a superstimulation treatment. Barros et al. (2008) conducted an experiment in which Nelore cows were superestimulated with Folltropin-V over 3 days and the last two FSH injections (on the fourth day) were replaced by injections of 200 IU of eCG. Donors in
the control group were superstimulated with the conventional treatment of eight twice–daily decreasing doses of FSH over 4 days. Treatment with eCG significantly increased (P <0.03) the number of ova/embryos collected (10.0 ± 1.5 vs 6.7 ± 1.2 for eCG-treated and the control cows, respectively) and numerically increased the number of transferable embryos (7.3 ± 1.2 vs 5.1 ± 1.1 for eCG-treated and the control cows, respectively). However, Sartori et al. (2009) found no beneficial effect of eCG in Nelore heifers.

To further investigate this alternative, we conducted an experiment with Brangus cows and heifers (Reano et al. 2009). Thirty-eight cows and 25 heifers were randomly allocated into three treatment groups. On Day 0, all donors received a progesterone releasing device and 50 mg progesterone and 2.5 mg EB im and superstimulation with Folltropin-V was initiated on Day 4. The donors of the Control Group received Folltropin-V in twice-daily decreasing dosages for 4 days (total dose 280, 320 or 400 mg NIH-FSH-P1). Donors received PGF2α in the morning and afternoon of Day 6 and progestin devices were removed in the afternoon of Day 7. All donors received GnRH in the morning of Day 8 and were FTAI 12 and 24 h later. Donors in the FSH+eCG Day 6 Group, received only the first four injections of Folltropin-V (on Days 4 and 5) and on Day 6, FSH injections were discontinued and replaced by a single injection of 400 IU of eCG (i.e. no Folltropin-V or eCG injections were given in the afternoon of Day 6 or on Day 7). The administrations of PGF2α, removal of the device, GnRH and FTAI were performed as in the Control Group. Finally, donors in the FSH+eCG Day 7 Group, received the first 6 injections of Folltropin-V (on Days 4, 5 and 6) and the last two injections of Folltropin-V on Day 7 were replaced with two injections of 200 IU eCG, given in the morning and afternoon of Day 6. The administrations of PGF2α, removal of the device, GnRH and FTAI were performed as in the Control Group. Ova/embryos were collected on Day 15.

As indicated in Table 6, treatment with two doses of eCG resulted in an increased number of transferable embryos. Furthermore, there were no differences between donors superstimulated with the twice daily injections of Folltropin-V over 4 days (Control) and those receiving Folltr opin-V for the first 2 days and then a single dose of 400 IU eCG on Day 6, when the first PGF2α was given. Undoubtedly these treatments are presented as a very interesting option that should be pursued further.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Total ova/embryos</th>
<th>Fertilized ova</th>
<th>Grade 1 &amp; 2 embryos</th>
<th>Grade 1,2 &amp; 3 embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>10.9±1.2a</td>
<td>7.4±1.1a</td>
<td>5.9±1.1a</td>
<td>6.4±1.1a</td>
</tr>
<tr>
<td>FSH+eCG Day 6</td>
<td>21</td>
<td>12.2±1.8ab</td>
<td>9.6±1.4ab</td>
<td>7.4±1.1a</td>
<td>8.0±1.2ab</td>
</tr>
<tr>
<td>FSH+eCG Day 7</td>
<td>21</td>
<td>15.6±1.6b</td>
<td>11.6±1.3b</td>
<td>10.1±1.1b</td>
<td>10.7±1.2b</td>
</tr>
<tr>
<td>P Value</td>
<td>0.10</td>
<td>0.08</td>
<td>0.03</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

Summary and conclusions

Although considerable progress has been made over the past several years, there is still a great need in the industry to simplify the superstimulation programs with an improvement in the number of transferable embryos collected per donor cow. Protocols that control follicular wave emergence and ovulation have provided a simple approach to superstimulate donor cows without depending on estrus detection. However, estradiol cannot be used in many parts of the world and mechanical removal of the dominant follicle requires the use of specialized equipment and trained technical staff. GnRH or pLH are other alternatives, but the efficacy of these treatments in inducing ovulation (and synchronization of follicle wave emergence) in groups of randomly cycling animals is not more than 60%. An alternative approach is to increase the response to GnRH by inducing a persistent follicle and initiating FSH treatments during the
first follicle wave after ovulation. To further simplify the superstimulation protocol, FSH can be mixed with a slow release formulation and administered in a single intramuscular injection without negatively affecting the number of transferable embryos. Finally, recent experiments have shown that it may be possible to increase the number and quality of embryos by the addition a small dose of eCG at the end of the FSH treatment. All these alternatives may provide new and efficient ways to facilitate widespread application of embryo transfer technologies.

Acknowledgments:
Research was supported by the Instituto de Reproducción Animal Córdoba (IRAC) and Bioniche Animal Health (Canada). We also thank Biotay SA (Argentina) for the provision on PGF$_2\alpha$ and GnRH and facilitating the importation of the other hormones used in these trials. Special thanks to our colleagues of IRAC for technical assistance.

References


OPPORTUNITIES FOR THE PRODUCTION OF RECOMBINANT GONADOTROPINS FOR ASSISTED REPRODUCTION AND EMBRYO TRANSFER

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Introduction

Pharmaceuticals have become a major part of human life in the treatment, management and prevention of illness and disease. Today, the term pharmaceutical is used in a broad sense to encompass therapeutic agents derived from both chemical-based (drugs) and protein-based (biologics) processes.

Biotechnology has become a household word which can be simply defined as harnessing a biological mechanism, usually a living system, to create a desirable outcome such as production, transformation, or elimination. With regards to pharmaceuticals, this means using a biological system to produce a product, typically a protein biologic, or to transform an existing chemical structure, thereby producing an active pharmaceutical ingredient (API); the active component of a drug. Examples of biological-based pharmaceuticals include vaccines, hormones and proteins, collectively known as biopharmaceuticals. Most of today’s pharmaceuticals utilize some aspect of biotechnology in their manufacture or testing.

The most important biopharmaceuticals used in animal reproductive technology are the pituitary glycoprotein hormones which include: follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and chorionic (placental) gonadotropins; which are produced exclusively in the mare (eCG) or women (hCG). Bioniche Animal Health is the world’s leading producer of veterinary hormones, supplying more than 80% of the FSH and LH used annually, and a significant supplier of eCG. The veterinary products currently available on the commercial market are all produced by extracting and purifying the desired hormones from pituitary tissue, blood or urine. Given the potential risk of pathogen transmission arising from the use of animal-derived products, it is foreseeable that regulatory authorities may soon require these products to be serum and animal-derived product free; thereby making recombinant production the most likely source of these hormones. Biotechnology brings the promise of animal-free, affordable products that mirror the native protein in structure and function.

Bioniche Animal Health is working with several biotechnology companies and universities around the world to develop novel expression technologies that will allow for the cost-effective production of high-quality, authentic, recombinant reproductive hormones for the animal industry. In this light, we will present the various biotechnology expression systems currently available, their advantages and disadvantages with regards to commercial production, and provide specific commentary as to the production of recombinant FSH and LH for veterinary applications.

Production

Commercial production of a recombinant biopharmaceutical protein requires the selection of an appropriate expression system, which is dependant on the nature of the protein being expressed. Critical factors to be considered in the selection of a pharmaceutical expression system include: accurate
production of the primary protein including disulphide bridges and conformational domains (1° and 2° structure), as well as correct post-translational modifications such as cleavage, glycosylation, phosphorylation, sulfation, myristoylation, etc., in order to form an authentic three-dimensional structure (3° structure). For proteins composed of multiple subunits, such as the gonadotropins, FSH and LH, the conditions must be suitable for these subunits to correctly associate with each other (4° structure) in order to form immunologically active epitopes and biologically functional protein complexes.

As the complexity of the protein structure increases, such as with glycoprotein hormones, the selection of an appropriate expression system becomes critical in order to create a functional and commercially viable recombinant product. In the case of reproductive hormones, FSH, LH and eCG, the quaternary (4°) structure is extremely important because these hormones are each composed of two non-covalently linked subunits, α and β. The α-subunit is identical for each of these hormones, and it is only the β-subunit which is different and determines the hormones’ distinctive properties (Pierce and Parsons, 1981). All of these subunits undergo critical post-translational glycosylation events in which specific carbohydrate moieties are added to the protein. It is the glycosylation events that ultimately determine the functionality of these hormones, including metabolic clearance rate (half-life) and receptor-binding ability (potency). Each of these hormones are glycosylated differently, e.g., the sulfated (SO₄) oligosaccharides of LH terminate in N-acetylgalactosamines (GalNAc), while FSH and eCG oligosaccharides are not sulfated and more commonly terminate in sialic acid and galactose (Gal) (Manna et al., 2002; Gordon, 2002).

No single expression system or cell type is capable of performing all of the possible post-translational modifications for every potential recombinant protein. Thus, it is typically necessary to evaluate a number of different expression systems in order to find one that can produce a biologically authentic product. Only after a functional expression system is identified do the issues of production cost and process engineering become relevant. If you cannot produce the correct product, the costs associated with the creation of a scalable and affordable expression system with an effective and economical downstream process (DSP) are purely academic.

**Bacterial Expression**

Bacterial expression systems are excellent candidates for the production of non-glycosylated proteins. Prokaryotic organisms such as *Escherichia coli* and *Salmonella typhimurium* have been used extensively for the expression of a wide variety of foreign genes and as a result, many production, stabilization, and optimization strategies have been described. A large number of veterinary biologics have and are being produced in prokaryotic systems, including Protein G of the respiratory syncytial virus expressed in *E. coli* (Murby, 1995; and Martin-Gallardo et al., 1993), antigens of feline leukemia virus expressed in *E. coli* (Marciani et al., 1991), and lipopolysaccharide (LPS) from *Shigella sonnei* expressed in *Vibrio cholerae* and *Bacillus brevis* (Nagahama et al., 1996).

While prokaryotic expression is efficient and affordable for the production of a broad range of immunogens, a number of natively glycosylated proteins, including FSH, LH, and eCG are not biologically active following production in prokaryotic expression systems due to the lack of glycosylation. This limits their ability to bind to receptor molecules, and increases their susceptibility to metabolic clearance. Additionally, the presence of lipopolysaccharides and other bacterial pyrogens may result in toxicological complications, interference and possible anaphylactic reactions. Therefore, eukaryotic expression systems such as yeast, insect cells, plants and mammalian cells are much more suited for the expression of glycoproteins (and other mammalian proteins) making them the predominant systems of choice.
Yeast Expression

Yeast has a long history of use in human medicine, and is extensively used in the production of food, alcohol and fuel. The most characterized yeast is *Saccharomyces cerevisiae* which was used to produce the first ever subunit vaccine for Hepatitis B (Valenzuela *et al.*, 1982) which was subsequently licensed and commercialized in 1986. From a commercial prospective, yeast expression systems such as *S. cerevisiae* or *Phichia pastoris*, are attractive due to the fact that their production facilities, cost, scale-ability, and ease of genetic modification is similar to bacteria-based systems. However, yeast systems have several distinct advantages over prokaryotes, including: the ability to express glycosylated protein, the absence of pyrogens, and low immunogenicity of the native yeast host cell. All of these factors combine to make yeast expression systems particularly attractive in producing antigens for veterinary biopharmaceuticals.

Many researchers have attempted to produce FSH and LH from many species using the methyltrophic yeast, *Pichia pastoris*, which is renown for its tendency to avoid over glycosylation, a characteristic common of other yeast expression systems (Table 1). Of particular interest is the use of fusion proteins to express the FSH alpha and beta subunits together, thereby ensuring stoichiometric availability and functional association of the subunits (Sen Gupta and Dighe, 2000; Fidler *et al.*, 2003; Kasuto and Levavi-Sivan, 2005).

<table>
<thead>
<tr>
<th>Species</th>
<th>Hormone</th>
<th>Reference</th>
<th>Yield</th>
<th>Bioactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine</td>
<td>FSH – coexpress αβ</td>
<td>Richard <em>et al.</em>, 1998</td>
<td>10 µg/mL</td>
<td>Yes</td>
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<tr>
<td>Ovine</td>
<td>FSH – α subunit</td>
<td>Fidler <em>et al.</em>, 1998</td>
<td>51 ng/mL</td>
<td>Yes</td>
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<tr>
<td></td>
<td>FSH – β subunit</td>
<td>Fidler <em>et al.</em>, 1998</td>
<td>17 ng/mL</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>FSH – fusion β-α</td>
<td>Fidler <em>et al.</em>, 2003</td>
<td>140 ng/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Bovine</td>
<td>FSH – β subunit</td>
<td>Samaddar <em>et al.</em>, 1997</td>
<td>200 ng/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Japanese eel</td>
<td>FSH – coexpress αβ</td>
<td>Kamei <em>et al.</em>, 2003</td>
<td>2 µg/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Tilapia fish</td>
<td>FSH – β subunit</td>
<td>Kasuto and Levavi-Sivan, 2005</td>
<td>4 µg/mL</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>FSH – fusion β-α</td>
<td>Kasuto and Levavi-Sivan, 2005</td>
<td>80 ng/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Human</td>
<td>CG – coexpress αβ</td>
<td>Sen Gupta and Dighe, 1999</td>
<td>3 µg/mL</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>CG – fusion α-β</td>
<td>Sen Gupta and Dighe, 2000</td>
<td>not reported</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>LH – coexpress αβ</td>
<td>Gadkari <em>et al.</em>, 2003</td>
<td>not reported</td>
<td>Yes</td>
</tr>
</tbody>
</table>

In an attempt to overcome the limitations of the yeast glycosylation system, several researchers are attempting to “humanize” the yeast host strains by introducing the genes coding for the specific enzymes leading to the various glycosylation patterns. Most notable is Tillman Gerngross of Dartmouth College (Hanover, New Hampshire) who has accurately introduced all human glycolsylation events, with the sole exception of sialic acid, into *Pichia* (Gerngross, 2004). The completion of this work may indeed open up the world of glycoproteins for cost-effective, large-scale production of veterinary reproductive hormones in yeast.

Insect Cell Expression

Insect cell-based expression systems may also produce glycosylated proteins; however glycosylation patterns are more comparable with those of higher eukaryotics than those produced by yeast. For this reason, it is believed that proteins produced in insect cells have greater biological value than the same protein produced in yeast. The insect cell expression system is based on the infection of cultured lepidopteran cells with a recombinant baculovirus designed to express the gene product under the control of a strong polyhedron promoter. This typically results in a high yield of immunologically active protein.
In 1999, Hu et al. described the baculovirus/insect cell expression of several structural proteins from the important poultry pathogen infective bursal disease virus (IBDV). Significant improvements in yields were subsequently obtained through the optimization of media, dissolved oxygen and the use of protease inhibitors to inhibit protein degradation.

The major limitation of insect cell expression has been the inability to achieve high densities due to the requirement of high dissolved oxygen levels combined with shear sensitivity. Typical oxygen supplementation methods employed in fermentation such as sparging and mechanical agitation induce significant shear, which limits cell densities. However, the recent development of synthetic serum-free media and lipid surfactants are allowing for large scale, high density fermentation of insect cells (Palomares et al., 2000; Ikonomou et al., 2001; Ikonomou et al., 2003). This may eventually lead to cost efficiencies sufficient for use in veterinary biologics.

To date, several groups have expressed FSH and LH in baculovirus systems. These recombinant proteins were shown to be biologically active; however, they did not possess the same glycosylation patterns as native hormones (Bozon et al., 1995; Kato et al., 1998; van de Wiel et al., 1998). Thus, while the glycosylation patterns of insect cells are more similar to mammals than those of the native bacterial or yeast systems, they are still insufficient for production of FSH, LH or eCG for veterinary reproductive practice.

Plant-Based Expression

Recently, plants have become the focus of a number of researchers in the development of biofactories for recombinant proteins and biological products (Hood and Howard, 2002; Carter and Langridge, 2002; Korban, 2002). Of interest is the expression of biopharmaceuticals, because plants have the ability to produce glycosylated proteins similar to that of higher eukaryotes, and plant products have the potential to be administered orally. This would thereby avoid the expense associated with downstream processing and purification. The use of a cereal or oilseed crops also presents the possibility of the product being stable at ambient temperatures, therein allowing for long term-storage without the need of refrigeration and cold-chain distribution. While the level of expression in plants varies greatly, it seems that how the expression product is regulated, and where the protein is targeted within the cell has the greatest impact on yield. For example, subunit B of the cholera toxin has been expressed in potato tubers at 0.3% of the total soluble protein when targeted to the endoplasmic reticulum (Arakawa et al., 1997), yet the yield was 4% of the total soluble protein when expressed in the chloroplast (Daniel et al., 2001). Correspondingly, subunit B of the heat-labile enterotoxigenic E. coli toxin has been expressed in corn seed at 0.3% of the total soluble proteins, while modification of the regulatory sequences has achieved up to 12% (Chikwamba et al., 2002, Streatfield et al., 2003). Further research may allow for the development of predictable and reliable high yielding expression constructs in the near future.

The development of plant-based, direct-fed biopharmaceuticals, such as vaccines, offers a considerable advantage when compared to the high cost of production, processing, formulation and delivery of conventional products. However, direct feeding is not an option for the production of gonadotropins, thus full scale extraction and downstream processing would be required. In 2001, a Dutch group successfully expressed an FSH β-α fusion protein in Nicotiana bethamiana, a species related to tobacco (Dirnberger et al., 2001). This construct was shown to be targeted to the periplasmic space and was reported to yield 3% of the total soluble periplasmic protein. This suggests that this process would be reasonable for large scale commercial production.

Currently, the greatest scientific concern regarding the use of plant-based biopharmaceuticals is the risk to the human food chain due to inadvertent cross-contamination. Despite the demonstration of feasibility, no plant based veterinary biopharmaceutical has yet been licensed.
Mammalian Cell and Viral Expression

Mammalian expression systems remain the preferred method of commercial production for native glycoproteins and numerous mammalian cell lines from many different species and tissues have been established for the expression and production of recombinant proteins. Although this is the most common system for the production of human biopharmaceuticals, because the precise protein product can generally be produced, minimizing the amount of non-specific glycoprotein isoforms can only be achieved by selecting the ideal species and tissue type for use as the cell line. It is because of the contaminating glycol isoforms that purification is so important in preparing finished human products which are required to have very accurate defined specific activities and biological function.

In mammalian expression, the gene for the target protein can either be cloned directly into the cell line’s genome under the control of a mammalian promoter or introduced via a viral vector. For the production of purified recombinant proteins, the specific term of viral expression is a misnomer in that the expression does not actually occur within the virus particle itself, but rather expression is driven by strong promoters belonging to the virus genome which was latently inserted into the mammalian genome, similar to insect cell expression as mentioned previously.

Many researchers have expressed FSH, LH, and both eCG and hCG in mammalian cell lines producing biologically active and authentic products. Recombinant human FSH has been produced in this fashion for many years, however, due to low yields and high manufacturing costs, it is still considered cost-prohibitive for veterinary use. Due to the expense involved, the few veterinary biologics manufactured in mammalian cells utilizing mammalian viruses are limited to vaccines where only small amounts of unpurified antigen are required per dose. One example is Aujeszky’s disease virus gp50 and gp63 glycoproteins produced via the swinepox virus (Van der Leek et al., 1994; and Sheppard, 1999). As most mammalian cell lines require fetal bovine serum (FBS) as part of the growth medium, the potential exists for adventitious agents and transmissible diseases to be present in the final product. Several synthetic formulations of FBS are commercial available; however, their compatibility with a given cell line must be assessed on a case-by-case basis and their inclusion in the growth medium typically requires re-optimizing the culture conditions, a long and expensive process.

Animal Based Expression (Molecular Pharming)

Recently there has been a great deal of progress made regarding the expression of novel proteins in the milk or eggs of agricultural species such as sheep, goats and chickens. Several companies are currently producing commercial products in these systems commonly referred to as molecular pharming or biopharming (Larrick, 2001). Other systems which have been proposed include blood, urine, and seminal plasma. While the specific cost of production is extremely low, given that production is not only continuous, but also generational, the actual cost of production is relatively high due to facility costs which include biosecurity measures to prevent internal contamination with zoonotic pathogens and diseases, as well as external contamination of the agricultural food chain. From a biosecurity and licensing perspective, these products must still be considered animal-derived. Additionally, the costs associated with generating the transgenic strain remain staggeringly high; these costs are expected to decrease drastically in the coming years (Keefer, 2004; Kues and Niemann, 2004).

It can be expected that the transgenic production of gonadotropins will be very difficult as these products may have a negative effect on production within the mature animal. That being said, human growth hormone has been expressed in the seminal vesicle epithelium of mice yielding biologically active hGH at up to 0.5 mg/mL within the semen (Dyck et al., 1999). Additionally, FSH from goldfish has been successfully expressed within a unique trout embryo biofactory developed by a Japanese group (Morita et al., 2004). While this recombinant hormone has been shown to be biologically active, further work must be done to establish the authenticity of its glycosylation patterns.
Production of Recombinant Gonadotropins for use in Animals

Recombinant FSH and LH have been developed by several groups around the world, but use in animal reproduction has been limited due to the short half-life of FSH, in particular, in the circulation. Repeated administrations are required to maintain the blood circulating levels and induce follicular development. Human chorionic gonadotropin has one of the longest circulating half-life of the all the glycoprotein’s due to the presence of a unique carboxyl-terminal peptide (CTP) on the beta subunit (Saal et al., 1991). Glycosylation of the CTP occurs at four O-linked carbohydrate attachment sites and increases the terminal (elimination) half-life of the hCG by reducing glomerular filtration within the kidney. The CTP has also been shown to increase the half-life of recombinant human FSH when covalently bound to the β-subunit (rFSH-CTP; Matzuk et al., 1990). Nevertheless, recent research has demonstrated that multiple N-linked glycosylation sites are more effective at inducing folliculogenesis in the mouse (Ruman et al., 2005) than commercial rhFSH, and have higher biopotency than O-linked FSH analogs in the rat (Weenen et al., 2004).

Other recombinant gonadotropin analogues have been tested in different animal models. Yoon et al. (2007) evaluated the efficacy of single chain recombinant equine luteinizing hormone (reLH) in shortening the time to ovulation in cycling mares, and to determine the effects of treatment on endogenous hormones and inter-ovulatory intervals. They concluded that reLH is a reliable and effective agent that does not alter endogenous hormone profiles or affect inter-ovulatory intervals. In another experiment (Lemke et al., 2008), single chain analogs of recombinant human FSH (rhFSH), human chorionic gonadotropins (rhCG) and dually active gonadotropin constructs (FcCGβα) were examined in sheep. It was concluded that single-chain FSH activity supports follicle development, but not estrogen production. on the contrary, a construct that incorporated the β domains from both hCG and FSH had dual activity, and the author concluded that the long-acting nature of the single-chain constructs suggest that these recombinant gonadotropins may be effective alternatives to pituitary- or placental-derived gonadotropins in out-of-season breeding and/or superovulation protocols (Adams and Boime, 2008).

Novel Recombinant Gonadotropin Analogs

Our co-development partner, Trophogen, previously described the first superactive analogs of glycoprotein hormones that increase receptor binding affinity and, both in vitro and in vivo biopotency (Szkudlinski et al., 1996; Grossman et al., 1998, Leitolf et al., 2000). This novel FSH analogue had higher efficacy than standard FSH in human cell lines, and in non-human primate studies showed no evidence of ovarian hyperstimulation at a presumably maximal dose, nor any evidence of immunogenicity. Using an HPLC-validated immunoassay, this analog has been shown to have high pharmacokinetic properties, both in rodents and monkeys, apparently as a result of delayed absorption. Using a retroviral bicistronic vector system, Trophogen has developed several stable Chinese Hamster Ovarian cell lines capable of producing high levels of recombinant glycoprotein hormone analogs and have optimized large-scale bioreactor production methods. In addition, they have developed high capacity purification methods suitable for commercial scale-up as well as rigorous assays for the quantification and characterization of purified analogs by multiple physicochemical methods, including carbohydrate analysis. Trophogen and Bioniche are collaborating in the development of a single injection form of their FSH analogue to be used in bovine superovulation.

In conclusion, the efficacy of several long-acting gonadotropins are being evaluated in models of animal reproduction. However, contrary to human medicine, where gonadotropins have been licensed for a number of years, none have yet been licensed for use in animals. It is reasonable to expect that in the near future recombinant hormones with long-acting activity will be licensed for use in veterinary medicine.
Summary

A great deal of potential exists in harnessing biotechnology in the development and production of high-quality non-animal-derived reproductive hormones. Active research and development projects for the production of FSH, LH and eCG in yeast, insect cell, mammalian and plant-based expression systems currently exist. With the continued development and humanization of the host strains used in these expression systems, the production of high-yielding, low-cost, biologically authentic rbFSH, rbLH and reCG is not far away. Bioniche Animal Health continues to be committed to providing high-quality veterinary hormones and is actively developing animal-product-free alternatives in order to maintain the supply of high-quality, safe products for use in the animal industry.

References


SMALL RUMINANT EMBRYO TRANSFER:
DONOR AND RECIPIENT MANAGEMENT

Hernan Baldassarre

Pharmathene Canada Inc., St-Telesphore, QC, Canada

Introduction & Presentation objectives

Donor Techniques & Management
• Superovulation
  • 101-Basics
  • Known influencing factors
  • Recommended protocol
• Embryo collection
  • Surgical
  • Non-surgical

Recipient Techniques & Management
• Synchronization protocols & Tips
• Embryo transfer protocols

LOPU-IVEP Overview
**Superovulation 101**

- Higher recruitment rate
- Lower regression rate

**If life was that simple..... BUT**

- Poor SOV response
- Low fertilization
- Premature Luteolysis

**UNPREDICTABLE RESULTS**

- Surgery
- Cost

- Low MOET utilization
Factors influencing **RESPONSE to SUPEROVULATION**

**HIGHER SUPEROVULATION RESPONSES** have been associated with:

- Animal in good body condition (not underfed or overfed)
- Middle age (not too young or too old)
- Reproductive season
- Good management (avoid all types of stress)
- Higher number of small follicles present in the ovary at superovulation start
- High progesterone at the start of superovulation
- Absence of large (dominant) follicles at start of superovulation

Some of these factors are quite **DIFFICULT TO CONTROL**

---

**Controlling PROGESTERONE LEVELS at start of FSH regime**

- Replacing the progesterone device with a new one at FSH start
- Initiating superovulation during luteal phase of a synch cycle.

![Diagram showing follicle counts and hormone levels](image)

- 9.6±0.6
- 8.3±0.7
- 6.8±0.5
- 4.1±0.6
Controlling the presence of a **LARGE FOLLICLE** at FSH start

- Mechanical ablation (e.g. by laparoscopy)
- Chemical ablation (e.g. estradiol administration)

\[ \frac{\text{Number of Large Follicles}}{\text{FSH Start}} = 6.1 \pm 0.9 \quad 11.6 \pm 2.0 \]

\[ \frac{\text{Number of Small Follicles}}{\text{FSH Start}} = 4.5 \pm 0.8 \quad 8.5 \pm 2.0 \]

Controlling number of **SMALL FOLLICLES** at FSH start

- GnRH agonists (based on pituitary depletion)
- GnRH antagonists (based on interference)
- Start superovulation on D1 (no dominance, only post-ovulatory follicle wave)
Factors influencing the impact of **POOR FERTILIZATION**

- Especially seen in GOOD responders
- Associated with poor semen transport in superovulated females
- Associated with big variation in timing of ovulation

**Solutions:**

- Intrauterine Insemination *(Evans & Armstrong, 1984)*
- Synchronize ovulation with GnRH *(Pierson et al., 2003; Baldassarre et al., 2004)*

Factors influencing incidence of **PREMATURE LUTEOLYSIS**

**Associated with:**

- Poor nutrition *(Jubbou et al., 1991)*
- Use of eCG in superovulation *(Pintado et al., 1990)*
- Stress *(Vivanco, 2001)*

**Strategies to prevent it:**

- Treatment with flunixin meglumine *(Bryce et al., 1988)*
- Avoid eCG in superovulatory treatments *(Armstrong & Evans, 1983)*
- Avoid persistent follicles (hCG-GnRH on day 3) *(Saharrea et al., 1998)*
- Recovery of oviduct-staged embryos
- Supplement P4 from shortly after ovulation *(Espinosa-Marquez et al., 2004)*
**Standard Superovulation PROTOCOL (sheep & goat)**

- **8 decreasing FSH injections**
  - 1.5, 1.5, 1, 1, 1, 0.5 and 0.3ml

- **-10d**
- **-48h**
- **-36**
- **-24**
- **-12**
- **0**
- **+12**
- **+24**
- **+36**
- **+48**
- **+60**
- **+8d**

- **P4-device IN**
- **Remove device**
- **+luteolytic**
- **GnRH**
- **Flushing**
- **Heat and AI**

- **Type of P4-device dictates need to change it @ FSH start (required for CIDR)**
- **FSH using step-down dosage**
- **Use GnRH @ 36h post sponge**
- **Day of flushing (stage of development of embryos)**

**Embryo Collection (Flushing)**

<table>
<thead>
<tr>
<th>Method</th>
<th>Recovery</th>
<th>Reliability</th>
<th>Repeatability</th>
<th>Other Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>SURGICAL</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>Flush of poor responders</td>
</tr>
<tr>
<td>SEMI-LAP</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>Expensive Equipment</td>
</tr>
<tr>
<td>LAPAROSCOPIC</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>Equipment + high skill</td>
</tr>
<tr>
<td>TRANSCERVICAL</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>Adult goats only, skill</td>
</tr>
</tbody>
</table>
Non Surgical (transcervical) flushing in goats

<table>
<thead>
<tr>
<th># of GOATS</th>
<th>BREEDING</th>
<th>TOTAL OVA</th>
<th>AVG OVA</th>
<th>TOTAL TE</th>
<th>AVG TE</th>
<th>% UFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>CERVICAL</td>
<td>335</td>
<td>7.6±6</td>
<td>179</td>
<td>4.1±5</td>
<td>42%</td>
</tr>
<tr>
<td>38</td>
<td>LAP</td>
<td>207</td>
<td>5.4±5</td>
<td>197</td>
<td>5.2±5</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>82</td>
<td>ALL</td>
<td>542</td>
<td>6.6±5</td>
<td>376</td>
<td>4.6±5</td>
<td>26%</td>
</tr>
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</table>

Recipient Synchronization PROTOCOL

<table>
<thead>
<tr>
<th>Days</th>
<th>Hours</th>
<th>Heat Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-10</td>
<td>-48</td>
<td></td>
</tr>
<tr>
<td>-24</td>
<td>0</td>
<td>Prostaglandin + eCG</td>
</tr>
<tr>
<td>+24</td>
<td>+36</td>
<td>Remove device</td>
</tr>
<tr>
<td>+48</td>
<td>+60</td>
<td>GnRH</td>
</tr>
</tbody>
</table>

Important FACTORS
- Good condition and health status
- Properly synchronized with donor (between 0-24h synchrony)
- Good husbandry to avoid stress (especially in goats = ERCL)
- Breed selection (facilitate parturition)
- Out-of-season ➔ replace GnRH with hCG (500Ui)
Embryo transfer TECHNIQUE

<table>
<thead>
<tr>
<th>Method</th>
<th>Reliability</th>
<th>Repeatability</th>
<th>Other Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical</td>
<td>+++</td>
<td>+</td>
<td>SX of culled recipients</td>
</tr>
<tr>
<td>Semi-Lap</td>
<td>+++</td>
<td>++</td>
<td>Equipment cost</td>
</tr>
<tr>
<td>Laparoscopic</td>
<td>++</td>
<td>+++</td>
<td>Cost, skill, Embryo loss</td>
</tr>
</tbody>
</table>

LOPU-IVP as an alternative to traditional MOET

Potential to produce more offspring from genetically valuable animals than standard MOET procedures

- Less invasive: More collections (pregnancies) per donor
- Avoid problems of poor response to superovulation
- Avoid problems associated with poor fertilization
- Avoid problems derived from premature luteolysis
- Opportunity for valuable animals that failed to produce by MOET
- Valuable animals that acquired fertility issues with age
- JIVET: Shorten the generation interval increase progress
Basic LOPU-donor treatment

Days
-10
-48
-36
-24
-12
0

Hours

MPA Sponge (Veranix®)

125 μg cloprostenol (Estrumate®) +
80 mg FSH (Folltropin-V®) +
300 IU eCG (Novormon®)

Remove sponge and LOPU

>400 procedures
4400 oocytes (10.5/ewe)

>2000 procedures
28,000 oocytes (14.0/goat)

LOPU-technique video

76
Summary of In vitro procedures

Immature oocytes (LOPU)

Maturation

Fertilization

IVM in TC199 supplemented with hormones + 100 µM

Cytosine + 10% FBS at 38.5°C in humidified incubator

with 5% CO₂ for 24-27h

IVF in TALP/SDF supplemented with 8g/L ESS at 38.5°C

in humidified incubator with 5% CO₂ for 15-20h

E.T. into D1 recipients

IVC to blastocyst stage

E.T. into D6 recipients

IVF \(\rightarrow\) The “bottleneck” in Small Ruminant IVEP

LOW Fertilization \(\leftrightarrow\) GOOD Fertilization \(\leftrightarrow\) Polyspermy

Semen

Batch

Dose

Serum

Batch

Dose

Capacitating Agents

Agent

Dose

Time of exposure
Recipient management & Embryo Transfer

- Recipients are synchronized with the same protocol referred previously
- Synchrony is aimed at recipient heat occurring synchronous with IVF
- Embryo transfer occurs normally around 72h post-sponge removal (1 day after OVL)

Prepubertal Response to Gonadotropin treatment

<table>
<thead>
<tr>
<th>Age</th>
<th>Adults</th>
<th>3–5 mo</th>
<th>2-3 mo</th>
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<td>✓R</td>
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### Prepubeal vs. Adult - Overall LOPU-IVEP Results

<table>
<thead>
<tr>
<th></th>
<th>Adult</th>
<th>Prepub</th>
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<td>42.5</td>
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<td>STGTT</td>
<td>311</td>
<td>38</td>
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### Reproductive Rescue Application of LOPU-IVEP

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<tr>
<td>ET Recipient</td>
<td>89% RR</td>
<td>72% F%</td>
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THE USE OF EMBRYO TRANSFER FOR THE INTERNATIONAL MOVEMENT OF BREEDING STOCK AND MANAGEMENT OF PATHOGEN INFECTION IN SWINE

Claire Plante and John Pollard
Genesafe Technologies Ltd.
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INTRODUCTION

Reproductive biotechnologies have both ignited the imagination and fueled the practical genetic advancements experienced by the livestock-breeding sector over the past century. Technologies such as artificial insemination, semen cryopreservation and embryo transfer associated technologies have facilitated both increased genetic selection and genetic resource availability, globally. Embryo transfer in particular has evolved, both in terms of its efficiency of use and in terms of its theoretical placement, to become much more then a method to increase the reproductive efficiency of donor females. Practically speaking, embryo transfer has matured to become a platform technology from which significant economic and biological advantages can be leveraged for commercial gain. Chief amongst these advantages, brought about by the use of embryo transfer, has been the economical movement of breeding stock in the form of embryos between distant localities or nations, and the significant decrease in the risk of pathogen transmission obtained during genetic movement with the use of cleansed embryos as the agent of genetic transfer. Such advantages hold significant promise in species such as swine, where breeding is organized hierarchically in production pyramids headed by a few large breeding organizations, where very large numbers of breeding animals are moved internationally on an annual basis, and where health holds such a determining role in the overall profitability of the industry. This promise is tempered, however, by the unique sensitivities of the porcine embryo which renders this species embryos much more vulnerable to degradation by exposure to an in vitro environment then when compared to bovine embryos at transferable stages of development. This sensitive nature, inherent in porcine embryos, has mandated the development of uniquely evolved commercial embryo transfer systems. Systems, wherein embryos are strictly maintained at body temperature and in a developing state during the entire recovery, manipulation, transport and transfer process. The following discussion will review the biological and clinical uniqueness of the porcine embryo and associated embryo transfer technologies as assessed through our commercial experience, clinical results utilizing embryo transfer for the international movement of swine breeding stock and pathogen elimination, as well as, outline possible scenarios for the use of embryo transfer at various levels of the swine production pyramid.

THE EARLY PORCINE EMBRYO

While the pre-implantation porcine embryo shares the same compelling beauty and general developmental pattern as embryos derived from all other livestock species, unique differences do exist which can significantly impact their handling requirements, morphological evaluation and clinical utilization in the field. Included amongst the unique characteristics possessed by early stage porcine embryos are differences in the stage, timing and sequence of critical developmental events, their cellular composition and resulting morphological appearance, specific cellular and environmental sensitivities that render porcine embryos uniquely vulnerable outside of the maternal system, and their capacity to migrate and reposition themselves within the uterine lumen. An awareness of these peculiarities is critical to the development of effective embryo transfer systems, which can maintain the viability of porcine embryos during international transport.
Morphology and Peculiarities

At first glance, those experienced with ruminant embryos will immediately be aware of the distinct darker coloration inherent in the porcine embryo. This coloration pattern, shared with embryos derived from certain carnivore species such as the canine, results from the inclusion of darkly colored lipid material in the cytoplasm of embryonic cells (Norberg, 1973). The accumulated dark lipid in the cytoplasm of oocytes and embryos is naturally occurring in all pre-hatching stage porcine embryos (as apposed to in vitro derived morulae and blastocysts in cattle, wherein the condition is an artifact of human manipulation) and results in several clinically relevant consequences such as the inability to observe pronuclei after fertilization in zygotes and a hyper-sensitivity to chilling injury which will be discussed later in this article. The cytoplasmic lipid material, which is normally equally distributed throughout the cytoplasm of individual embryonic cells, can be displaced to the periphery of the cell without developmental consequence by high-speed centrifugation (Nagashima et al, 1994). The dark coloration of the porcine embryo does not result in a raged or unthrifty appearance in the developing embryos (as in the case of certain in vitro derived embryos) and normal morphological evaluation procedures can be applied to the porcine embryo.

Other unique morphological characteristics which have impact on the clinical assessment of porcine embryos intended for commercial transfer, include: a propensity of unfertilized ova to cleave and present a normal morphology through the second cleavage division when recovered during the first five days following ovulation induction; entrapment of many supernumery sperm in the zona pellucida of fertilized embryos; compaction at early cleavage stages (4-8 cell transition); and presentation of a hyper-compacted morphology in morulae that can result in the incorrect morphological classification of compact morulae as zygotes or unfertilized ova. The cleavage of unfertilized ova can present significant difficulty to embryologists inexperienced in evaluating porcine embryos. In contrast to ova derived from other livestock species, unfertilized oocytes recovered from porcine donors several days after ovulation are often found to have cleaved to the first or second cleavage division, thus making the determination of fertilization by cleavage alone very unreliable. A unique character of the porcine zona pellucida that can assist with the evaluation of fertilization in early cleavage stage embryos is the manifestation of a partial zona reaction in porcine zygotes at fertilization (Hunter, 1991). This partial zona reaction in the fertilized zygote results in the hardening of only the inner half of the zona pellucida to sperm penetration. Thus, early fertilized porcine embryos usually collect many hundreds or even thousands of supernumery sperm that have partially penetrated and thereafter become entrapped in the outer surface of the zona pellucida. Conformation of fertilization in early stage porcine embryos can therefore be confirmed by the presence of supernumery sperm entrapped in the outer surface of the zona pellucida of early cleaved embryos and not by cleavage alone. Using supernumery sperm to confirm fertilization in early cleavage stage embryos has been highly effective in our clinical experience.

Pre-hatching stage porcine embryos also exhibit exquisite sensitivity to certain environmental factors, which must be acknowledged and understood by the clinical embryo transfer team in order to achieve maximal success. In contrast to ruminant embryos which when collected from the uterus (morulae and blastocyst stage embryos) exhibit no apparent sensitivity to chilling above the point of cellular freezing, porcine embryonic cells are the most sensitive mammalian cell type yet studied in relation to chilling sensitivity at relatively high temperatures (Polge et al., 1974; Plante et al., 1993; Pollard and Leibo, 1994). Porcine embryos at any stage of development, prior to hatching as blastocysts, can be lethally damaged by exposure to temperatures as high as +14°C. Even short exposures to +14°C (3-5 minutes) will result in the majority of embryos exhibiting total cellular lysis within a matter of hours. This effect is highly precise in terms of the initiating temperature (embryos exposed to +15°C survive while embryos exposed to +14°C or below perish) and is repeatable amongst all tested genetic strains. Exposure to these relatively high lethal temperatures appears to result in a cascading effect, which results in cytoplasmic lysosome release and total cellular degradation. The effect also appears to be related to the presence of intracytoplasmic lipids as removal of lipids by high-speed centrifugation significantly changes
the thermodynamic nature of chilling injury in early stage porcine embryos (Nagashima et al., 1994). One effect of this extreme sensitivity to chilling injury is the requisite need to maintain high ambient temperatures throughout the entire embryo transfer process (during surgical embryo recovery, manipulation, culture and surgical or non-surgical transfer procedures). Scrupulous attention to temperature maintenance during the embryo transfer process has resulted in a significant reduction in the variance experienced in embryo transfer results in our clinical practice utilizing the technology. When viewed from this perspective, the general failure experienced during early attempts at the international movement of porcine embryos for which the transport temperature was not precisely controlled during shipment or wherein embryos were deliberately chilled during transport can be readily understood. The extreme chilling sensitivity of transferable stage porcine embryos has also resulted in the failure to date for the development of commercially viable cryopreservation systems for use with pre-hatching stage porcine embryos. Despite an enormous research effort directed at the development of cryopreservation systems for porcine embryos, and limited laboratory success utilizing ultra-rapid freezing techniques and highly laborious manipulations, no commercially viable cryopreservation system has yet proven itself reliable in the clinical setting. The inability to cryopreserve porcine embryos is a significant impediment to the extensive commercial utilization of embryo transfer technology in swine and has mandated the evolution of embryo transfer systems, which maintain porcine embryos at body temperature and in a growing state. The need to maintain porcine embryos in a growing state when outside the maternal system has thus required the development and use of specialized media and transport incubators that can competently maintain normal developmental viability of cultured embryos.

**Developmental Pattern**

The early porcine embryo, as for embryos from all other mammalian species, comes to be and initiates development in the oviduct, and thereafter passes into the uterus around the time of cellular compaction. In swine, the first and second embryonic cell cycles transpire within the lumen of the oviduct in close association with the mucosal epithelium (Hunter, 1974) and are characterized by reductive divisions, which proceed under the sole control of stored maternal mRNA (Jarrell et al, 1991). Initial transcription of the embryonic genome occurs relatively early in the porcine embryo during the second cell cycle (Jarrell et al, 1991). The developing swine embryo enters the uterus at the 4-cell stage through the funnel shaped utero-tubal junction on the third day of the estrous cycle (Hunter, 1974). Morula undergo cellular compaction during the transition from the second to the third cell cycle, which leads to development of distinct inner, and outer cell groups (Hunter et al, 1974) and is followed by the formation of the fluid-filled cavity within the embryo. Formation of this blastocoelic cavity coincides with the initial differentiation of epithelial cells from pluripotent embryonic cells (Ducibella et al, 1975) and denotes transformation from the compact morula to blastocyst stage. Cellular differentiation and cavitation occur around the fifth day after estrus (Hunter, 1974). The early and expanded blastocyst is comprised of outer differentiated trophoderm cells and a group of pluripotent inner cells, the inner cell mass, which they completely surround (Ducibella et al, 1975). Between days 6 and 7 after estrus the expanding blastocyst hatches free from the zona pellucida and thereafter forms a spherical embryo prior to elongation on day 12 (Geisert et al, 1982a). By day nine of development, the developing porcine blastocysts initiate production and secretion of estradiol from their trophoderm cells. Estradiol secreted by the spherical embryos in turn signals their presence to the maternal system and functions to inhibit luteolysis and thus establishes pregnancy through the long-term maintenance of luteal function and progesterone production (Geisert et al, 1982b). It is generally believed that the presence of a minimum of 4 embryos is required to establish pregnancy around day twelve of development (Senger, 1997). Porcine embryos also possess the unique capacity to migrate and reposition themselves so to spread themselves equally throughout the entire available uterine lumen in the host gilt or sow (Dhindsa et al, 1967; Anderson and Parker, 1976). The ability of embryos to migrate and reposition themselves throughout the uterine lumen in both available uterine horns results in the clinical advantage of having to deposit embryos in only a single uterine horn in order to establish a normal and maximally productive pregnancy in recipient females.
Advantageous Characteristics

Size

Pre-hatching stage porcine embryos possess several unique characteristics that make them effective for use in facilitating international genetic movement and health related biosecurity. Amongst these beneficial characteristics are that pre-hatching stage embryos are very small in size (averaging ~ 150 mm in diameter) and are encased within an impermeable protective shell. While it is confined within the zona pellucida, mammalian embryos remain very small and are therefore very economical to transport to distant locations. It is this significant size advantage of the embryo that in fact grants embryo transfer systems their principle economic advantage over conventional live animal transport systems. In swine embryo transfer, many hundreds of embryos can be transported in a transport incubator the size of a brief case for the cost of a single airline ticket. An entire herd of swine can thus be shipped using these methods to any location across the globe.

Pathogen Defenses

While in actual practice, donor females and selected sires are thoroughly tested for their health status prior to acceptance in export programs, biologically speaking the disease status of the donor sow has no impact upon the disease status of piglets derived from embryo transfer if proper embryo washing procedures are adhered to during the transfer process. This lack of health status relationship between donor sows and their resulting offspring derived from embryo transfer arises from the protective barrier formed by the zona pellucida. The zona pellucida completely encases the developing embryo over the first six days of life so to create an impermeable barrier (if intact) to pathogens. Because of the impermeable nature of the zona pellucida, only those pathogens that can adhere to the outside surface of the zona pellucida could be transmitted with the embryo during the transfer process. The inherent safety afforded by embryo transfer in the elimination of disease causing environmental pathogens stems from the fact that very few pathogens can adhere to the zona pellucida in sufficient number so to constitute an infective dose during transfer, and that those viruses that can adhere to the zona pellucida (i.e. Pseudorabies Virus) are very susceptible to trypsin digestion. Strict adherence to the International Embryo Transfer Society embryo washing procedure eliminates virtually any potential for pathogen transfer during embryo transfer. Embryo washing technologies have been experimentally shown to eliminate African Swine Fever Virus, Foot and Mouth Disease, Hog Cholera Virus, Swine Parvovirus, Porcine Reproductive and Respiratory Syndrome Virus, Pseudorabies Virus, Swine Vesicular Disease Virus, and Vesicular Stomatitis Virus from embryos recovered from infected donors (Singh, 1993; Plante et al, 1997; Randall et al, 1999). Possible exceptions to the above mentioned pathogen defense mechanisms inherent in the architecture of the pre-hatching stage embryos include retro-viruses that are already incorporated into the DNA of oocytes or sperm prior to fertilization, and Porcine Circovirus II which is currently being investigated by Genesafe in collaboration with Dr. Andrea Belinski of the CFIA.

Embryo transfer is currently the only known technology that can effectively eliminate viruses (Parvovirus and PRRSV) that cross the placenta during fetal development in infected populations. Such viruses cannot be eliminated by traditional cesarean based gnotobiotic piglet production systems and only embryo transfer systems which function to cleanse contaminated embryos have the potential to eliminate pathogen infection on a biological basis in such infected populations. Embryo transfer can function to eliminate specific pathogen contamination in infected populations by combining embryo decontamination procedures with the intentional transfer of cleansed embryos into recipients selected to be naive for the pathogen(s) in question. Strict adherence to disease testing of donors and sires and to internationally approved embryo washing procedures has produced a system for the international exchange of breeding stock that decreases the risk of pathogen transfer to its lowest possible level.
The disease status of the offspring produced by embryo transfer is ultimately determined by the disease status of the recipient sows used to produce the piglets. By manipulating the disease status of the recipients, any disease status can be achieved in an intentional and sustainable manner. Through embryo transfer, piglets that are pathogen-free can be produced from any genotype if disease-free recipients are used. If disease-free piglets are not desired, recipients of any specific pathogen status can be successfully used. In areas or regions of high disease risk, embryos can be transferred into either naturally immune or vaccinated recipients such that piglets will acquire immunity through antibody transfer from the recipient.

Evolved swine embryo transfer systems thus have the potential to offer the swine breeding industry, for the first time in its history, a tool by which both the genetic composition and health status of breeding stock can be intentionally selected and controlled simultaneously to meet industry demand. The potential power of embryo transfer within modern swine production thus rests upon the two technological pivot points of donor and recipient selection. Genetic composition of the embryo transfer offspring will be completely determined by donor and sire selection, while the health status of the embryo transfer progeny will rest entirely with the health status of the selected recipient. By putting these two pivot points together within an integrated production system, embryo transfer will permit swine breeders to intentionally and simultaneously manipulate both the genetic composition and health status of the swine under their care. The overwhelming importance of health to the profitability of the swine industry is a powerful agent purposing the evolution of vision for the use of embryo transfer technologies in this species, as well as others.

**EMBRYO TRANSFER IN SWINE**

**Methods**

Embryo transfer is the process by which embryos are recovered from one female (the donor), before their attachment to her uterus, and thereafter transferred to the reproductive tract of another female (the recipient) for full term development to live offspring. The success of embryo transfer systems is dependent upon the recovery of viable embryos from the donor, the maintenance of embryonic viability during embryo manipulation, storage and transport to the site of transfer, and proper recipient preparation and transfer methodologies so to establish high pregnancy and offspring production rates. In addition, swine embryo transfer differs somewhat in focus from that of other livestock species in that constant attention must be paid, both in planning and implementation, to biosecurity and the risk of pathogen transfer. In our practice, this has lead to the development of completely separate embryo recovery and transfer teams, the scrupulous separation and disposal of all used equipment, use of all disposal products when available and lengthy time intervals between embryo transfer events.

In swine, embryo recovery must be conducted surgically due to the anatomical restrictions of the species, and as stated previously, embryos must be manipulated and transported at body temperature to sustain high viability. Though non-surgical transfer systems have been developed by the research community, all commercial embryo transfer in swine is currently conducted surgically because of its greater production capacity and reliability. The swine industry is unique in its sensitivity to production numbers and its expectations of reproductive technologies are very high in terms of expected pregnancy rates and litter size. Because of the inherent fertility and resident productivity of modern swine genetics, the industry is very intolerant of technologies that result in pregnancy rates and litter sizes below production standards. This implies, in our experience, that pregnancy rate averages below 70% and significant reductions in litter size will not be tolerated. This has lead to the stringent testing of reproductive technologies before commercial acceptance and a time lag between laboratory innovations and their subsequent commercial application. For certain technologies such as embryo cryopreservation, its widespread use may be delayed until such time when some as yet undiscovered technological breakthrough will overcome the natural vulnerability of porcine embryos to chilling injury. In the case of
non-surgical transfer technologies, it is perplexing why the technology has proven so unreliable in terms of its capacity for pregnancy establishment and piglet production under field conditions.

During the process of swine embryo transfer, donors are hyper stimulated (superovulation) following weaning, estrous synchronization by progestin treatment or abortion, or in prepubetal gilts. Superovulated donors are then artificially inseminated with pathogen-tested (PCR) semen from approved boars at estrus. Swine embryos are recovered over the first five days following ovulation so to assure that embryos are collected while still contained within the zona pellucida. Swine embryos are recovered surgically from either gilts or mature sows, or at the time of slaughter from prepubertal gilts. Surgical embryo recovery from genetically valuable donors requires rigorous adherence to sterile surgical technique and the use of advanced procedures to mitigate adhesion formation, as swine are exceptionally vulnerable to adhesion formation involving the reproductive tract (similar to the vulnerability of horses or humans in this regard). Recovered embryos are washed utilizing the approved protocol of the International Embryo Transfer Society. Current Canadian and International protocols allow no more than fifteen embryos to be washed together as a group during decontamination procedures. Embryos washed and treated in such a manner may be certified free of all known porcine pathogens. Because of their vulnerability, careful attention must also be paid to the maintenance of embryonic viability throughout the entire recovery, manipulation, transport and transfer process. For porcine embryos, very few media exist which can maintain embryonic viability beyond a twelve-hour period (Martin, 1986). Such a media has been developed by Genesafe over the past decade using Simplex optimization technologies with the focus of the optimization centered on pregnancy establishment and litter size using fixed transfer parameters and culture periods. The clinical results presented in this report have all been generated utilizing the Genesafe Transfer Solution (GTS) and portable shipping incubators (Minitube). Porcine embryos can be successfully maintained within the GTS system for at least 42 hours without diminishment in pregnancy rates or litter size following transfer (table 1). GTS allows for development from zygote to expanded blastocyst in an air environment and does not permit hatching of expanded blastocysts.

In addition to the culture media, careful attention must also be made to maintaining the environmental temperature as close to normal body temperature as possible throughout the entire process. Careful attention to temperature maintenance has greatly increased the repeatability of Genesafe results, though the comfort of the surgical recovery and transfer teams is somewhat compromised by these demands.

Ultimately, groups of embryos are transferred to synchronized recipients using simplified surgical transfer protocols. Recipients can be synchronized by weaning or can be prepared by either progesterone treatment or abortion. Best results are obtained when recipients are prepared to ovulate 24 to 48 hours after the ovulation of donors, if embryos are held more than 24 hours in transport. An asynchrony up to 72 hours is acceptable in swine.

Post-transfer recipient handling is also critically important to the success of the transfer process. Recipients should be maintained for the first 40 days post-transfer either in individual gestation crates or in stabilized social groupings established well in advance of the transfer. Maintenance of established social groups or individual housing of recipients is critical to the successful establishment of pregnancy by eliminating the social unrest and associated stress experienced with the remixing of sows or gilts into new social groupings. Mixing of recipients into unfamiliar social groups immediately after transfer can result in significantly reduced pregnancy rates and litter size.
Finally, methods used for the commercial movement of swine breeding stock must always focus on lowering the risk of pathogen transfer to their lowest possible level. Embryo transfer has great potential in this area, but proper clinical management procedures must always be adhered too for this potential to be realized under field conditions. A central focus on alleviating any potential for pathogen transfer has lead Genesafe to develop totally separate embryo recovery and transfer teams, strict policies relating to equipment sterilization and handling, rigorous compliance in the use of embryo evaluation and decontamination procedures, as well as self imposed adherence in maintaining lengthy time intervals between separate embryo transfer events. The implementation and strict adherence to these procedures has significantly enhanced the biosecurity of the evolved embryo transfer system under field conditions.

International Movement of Swine Breeding Stock using Embryo Transfer

Over the past decade Genesafe has collaborated with many different breeding companies to facilitate the international movement of breeding stock via embryo transfer. Embryos have been successfully transported using the evolved culture-transport system from North America to Europe, from Europe to North America, from the United Sates to Canada, and between different member states within the European Union. Example results from these embryo-facilitated exports are presented in Table 2 (data presented only from those clients wiling to share data publicly). Results demonstrate that commercial embryo transfer systems can be used to successful manage the international movement of swine breeding stock in an effective manner. No detectable pathogens were transferred from donor to recipient herds during these events and embryo transfer derived progeny have not presented increased incidents of malformations or health complications. Sporadic dystocia (< 0.2% of litters) has been experienced in recipients carrying very small litters at term (particularly in recipient carrying embryos from heavily muscled terminal sire lines).

Table 1. Pregnancy rate and litter size following 24, 42 or 72 hours of in vitro. culture in GTS*

<table>
<thead>
<tr>
<th>Culture Treatment</th>
<th>Number of Recipients</th>
<th>Pregnancy Rate (%)</th>
<th>Litter Size (Total Born)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>114</td>
<td>83%</td>
<td>9.4</td>
</tr>
<tr>
<td>42 hours</td>
<td>102</td>
<td>75%</td>
<td>9.1</td>
</tr>
<tr>
<td>72 hours</td>
<td>77</td>
<td>52%</td>
<td>7.3</td>
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</table>

* Genesafe Transfer Solution
Given appropriate synchrony and fertility in selected recipients, the overall productivity in swine embryo transfer is governed by both the developmental viability of the embryos transferred (their capacity to both establish pregnancies in the host recipients and their individual capacity to continue development into fully term piglets) and the total number of embryos transferred. Being a multiovulatory-litter being species, decisions related to the number of embryos transferred per recipient are more complicated than when compared to single ovulatory species where a one-to-one embryo:recipient ratio is the rule. Earlier research now supported by our clinical experience suggests that while pregnancy rates are maximized by the transfer of 15 embryos, that litter size continues to increase until reaching its zenith with the transfer of 25 embryos. Management decisions related to maximizing piglet production during a single embryo transfer event is thus governed by the availability of fertile synchronized recipients, total transferable embryo number and the interests and objectives of the particular client in question. The field data presented in this report represents an amalgam of embryo transfer events, which may have differed significantly in terms of the number of embryos transferred per recipient.

Clients report significant benefit from the use of embryo transfer for the safe movement of breeding stock internationally. These reported benefits include: significant lowering of the risk of pathogen importation into nucleus herds and the associated opening of closed nucleus herds to the import of new genetic lines; rapid introduction and dispersal of new genetic lines across the production pyramid and geographic borders; opening new international sales regions by the creation of new biosecure nucleus breeding units in geographically distant locations.

**Table 2. Results of the international movement of swine breeding stock using embryo transfer.**

<table>
<thead>
<tr>
<th>Export-Import</th>
<th>Number of Transfers</th>
<th>Mean Interval (h) Recovery-Transfer</th>
<th>Pregnancy Rate (%)</th>
<th>Litter Size (Total Born)</th>
</tr>
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<tbody>
<tr>
<td>North America - Europe</td>
<td>103</td>
<td>38h</td>
<td>79%</td>
<td>8.7</td>
</tr>
<tr>
<td>Europe - North America</td>
<td>181</td>
<td>42h</td>
<td>78%</td>
<td>8.1</td>
</tr>
<tr>
<td>Between EU Nations</td>
<td>59</td>
<td>24h</td>
<td>86%</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Pathogen Elimination Through the Use of Embryo Transfer in Swine

The first substantive use of embryo transfer in swine for the intentional elimination of specific contaminating pathogens occurred during outbreaks of Psuedorabies virus in North America (James et al, 1983). Currently, embryo transfer is again being used to deliberately decontaminate breeding stock infected with economically important pathogens such as Mycoplasma and PRRSV. Representative results from this work are presented in Table 3 and demonstrate that embryo transfer technologies can be used to effectively eliminate either PRRSV or Mycoplasma from infected stock as no cellular infection of either PRRSV or Mycoplasma has been detected in either embryo transfer derived piglets or their host recipients following the transfer of washed embryos.

Production results from genetic rescue projects involving the transfer of decontaminated embryos from infected donors into pathogen free recipients can be slightly reduced in terms of pregnancy rates and litter size. Such reductions are dependent upon the pathogens in question, the clinical status of the donors, the timing of the embryo transfer event, and the effect of all of these parameters on embryo viability.
Despite these potential challenges, reductions in overall production are generally slight and the ability to rescue valued genetics from infected nucleus units is of significant economic value to breeding companies.

Continued research efforts are needed in this area to define the capacity of embryo transfer technologies to mitigate the transmission of new infectious pathogens such as Circovirus, as well as to optimize the technologies use for specific pathogen elimination and treatment.

**Table 3.** Results on the use of embryo transfer for the elimination of porcine pathogens from infected breeding stock.

<table>
<thead>
<tr>
<th>Donor Health Status</th>
<th>Number of Transfers</th>
<th>Pregnancy Rate (%)</th>
<th>Litter Size (Total Born)</th>
<th>Total # Piglets Born</th>
<th>ET Piglet Health Born</th>
<th>Recipient Health Born</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor Herd 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRRSV (positive)</td>
<td>130</td>
<td>70%</td>
<td>7.8</td>
<td>710</td>
<td>Pathogen Free</td>
<td>Pathogen Free</td>
</tr>
<tr>
<td>Mycoplasma (positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor Herd 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRRSV (negative)</td>
<td>192</td>
<td>76%</td>
<td>8.4</td>
<td>1218</td>
<td>Pathogen Free</td>
<td>Pathogen Free</td>
</tr>
<tr>
<td>Mycoplasma (positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Use of Embryo Transfer in Commercial Swine Breeding**

Embryo transfer is evolving uniquely in the swine industry to constitute a technology platform that can benefit the industry in areas related to the economic and biosecure movement of breeding stock and the intentional upgrade of the health status of entire production pyramids. Such changes in the purposed use of this technology have been catalyzed by the unique importance of health to the profitability of the swine industry as a whole and the international structure of its breeding companies. Embryo transfer is already functioning to the benefit of this industry in a number of important ways. These include: the rapid and safe distribution of new genetics lines across geographically distributed nucleus breeding units, economic savings through the use of microscopic embryos as the agent of genetic movement, decreased risk of pathogen transfer during genetic movement, the development of open nucleus breeding programs, and the specific and controlled elimination of pathogens from infected populations. Because of its unique nature the swine industry may in fact have the capacity to push the evolution of embryo associated technologies in a manner which may benefit the entire livestock breeding community by focusing the future development of embryo transfer on its potential for health promotion and disease elimination.
References


Introduction

While genetics is the study of the hereditary transmission of traits expressed by individuals, genomics is concerned with the study of their DNA. The genetic merit of an animal depends upon its genes and how they are expressed. All genes are composed of molecules of DNA. The complete set of DNA for each animal (what we call its genome) is contained in the chromosomes of everyone of its cell, and can be obtained from any animal tissue.

Some traits are affected by a single change or mutation on one gene. Examples of this are CVM, BLAD or the red coat color in Holsteins. However, most of the traits we want to select for, such as production, conformation, longevity, reproduction and health, are influenced by small variations in a very large number of genes. One cannot select effectively for these traits by testing an animal’s DNA for one or two genes, as some genomics companies currently do. Instead, one must select for all genes affecting the trait at the same time. This is what genomic selection does. Genomic selection uses many genetic markers, i.e. molecules at specific points on the chromosomes where natural variation is known to exist, to tag each small region of the animal’s genome and account for all of the genetic variation in these regions (Figure 1). Then it becomes possible to predict an animal’s genetic merit for the complex traits we select for based on its DNA profile, i.e. the list of DNA markers carried by the animal.

Figure 1: Genetic markers and genes

In short, genomic selection consists in using DNA profiles to help predict the genetic merit of animals, and select them on that basis. It can be used for any trait for which traditional genetic evaluations are already computed.
The results

Semex has been a participant in various genomics research projects in Canada and abroad (Table 1).

Table 1: Genomics research projects to which Boviteq/Semex participated over the last few years

<table>
<thead>
<tr>
<th>Years</th>
<th>Collaborators</th>
<th>Objective of the project</th>
<th>Number of individuals tested</th>
<th>Number of markers per individual</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999-2006</td>
<td>Agriculture Canada (Lennoxville)</td>
<td>Assisted selection</td>
<td>1000 sires</td>
<td>155 *</td>
</tr>
<tr>
<td>2005-2007</td>
<td>Holstein Canada and others</td>
<td>DNA collection</td>
<td>3100 cows</td>
<td>55 *</td>
</tr>
<tr>
<td>2006-2007</td>
<td>University of Alberta</td>
<td>Assisted selection</td>
<td>380 sires</td>
<td>1 536**</td>
</tr>
<tr>
<td>2003-2007</td>
<td>University of Guelph</td>
<td>Assisted selection</td>
<td>800 sires</td>
<td>10 000**</td>
</tr>
<tr>
<td>2007-2008</td>
<td>USDA and others</td>
<td>Assisted selection</td>
<td>5 000 sires</td>
<td>50 000**</td>
</tr>
<tr>
<td>2003-2008</td>
<td>DairyGen</td>
<td>Genomic projects</td>
<td>Variable</td>
<td>Variable</td>
</tr>
</tbody>
</table>

Type of markers: *microsatellites; **SNP

Among these was a large North American genomic selection project led by the USDA. This North American project led to the development of a DNA chip with 60,000 well-spaced markers, constituting the best platform for genomic selection available to-date. More than 5,000 bulls, including about 1,500 from Semex, were genotyped. The experimental design for the project is shown in Table 2.

Table 2: North American project – Number of proven bulls used in the experimental design

<table>
<thead>
<tr>
<th>Breed</th>
<th>HO</th>
<th>JE</th>
<th>BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predictor bulls:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulls born &lt;1999</td>
<td>3576</td>
<td>743</td>
<td>225</td>
</tr>
<tr>
<td>Cows with data</td>
<td>202</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predictee bulls:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulls born &gt;1999</td>
<td>1759</td>
<td>425</td>
<td>118</td>
</tr>
</tbody>
</table>

Data from 2003 used to predict independent data from 2008
The key results from the project are shown in Tables 3 and 4. In the Holstein breed, the use of genomic information combined with the parent average increased the accuracy of evaluation substantially over that of the parent average alone.

Table 3: North American project – Gains in reliability above parent average by breed for yield traits (February 2009 validation)

<table>
<thead>
<tr>
<th>Trait</th>
<th>HO</th>
<th>JE</th>
<th>BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>24</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Fat</td>
<td>32</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Protein</td>
<td>24</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fat %</td>
<td>50</td>
<td>36</td>
<td>10</td>
</tr>
<tr>
<td>Protein %</td>
<td>38</td>
<td>29</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 4: North American project – Gains in reliability above parent average by breed for health and type traits (February 2009 validation)

<table>
<thead>
<tr>
<th>Trait</th>
<th>HO</th>
<th>JE</th>
<th>BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production life</td>
<td>32</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Somatic cell score</td>
<td>23</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Daughter pregnancy rate</td>
<td>28</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Final score</td>
<td>20</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Udder depth</td>
<td>37</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>Foot angle</td>
<td>25</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Stature</td>
<td>34</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

However, the resulting evaluations are far from being as accurate as bull proofs. For example, for protein and fat yields, using genomic data is equivalent to having between 9 and 18 daughters in addition to the parent average, instead of 80 to 100 daughters for a bull proof (Table 5). A good way to understand reliability is to look at the expected range of differences between the true genetic value of a bull and its estimate. Taking protein yield and over-all conformation as examples, these ranges are shown in Table 6 for reliabilities corresponding to parent averages, genomic evaluations and proofs.

Table 5: North American project – Daughter equivalents from genomic selection (from February 2009 validation)

<table>
<thead>
<tr>
<th>Trait</th>
<th>Parent average reliability</th>
<th>GPTA reliability (observed)</th>
<th>Additional daughter equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk yield</td>
<td>38</td>
<td>64</td>
<td>13</td>
</tr>
<tr>
<td>Fat yield</td>
<td>38</td>
<td>70</td>
<td>19</td>
</tr>
<tr>
<td>Protein yield</td>
<td>38</td>
<td>62</td>
<td>12</td>
</tr>
<tr>
<td>Productive life</td>
<td>33</td>
<td>65</td>
<td>67</td>
</tr>
<tr>
<td>SCS</td>
<td>34</td>
<td>57</td>
<td>26</td>
</tr>
<tr>
<td>Fertility (DPR)</td>
<td>32</td>
<td>59</td>
<td>98</td>
</tr>
<tr>
<td>Final score</td>
<td>29</td>
<td>49</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 6: Reliability versus confidence interval

<table>
<thead>
<tr>
<th>Reliability</th>
<th>Confidence interval¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (kg)</td>
</tr>
<tr>
<td>Parent average (PA): 35 %</td>
<td>33</td>
</tr>
<tr>
<td>Genomics +PA: 60 %</td>
<td>26</td>
</tr>
<tr>
<td>Genomics +PA: 70 %</td>
<td>23</td>
</tr>
<tr>
<td>Bull proof – 100 daughters 90 %</td>
<td>13</td>
</tr>
<tr>
<td>Bull proof – 100 daughters 95 %</td>
<td>4</td>
</tr>
</tbody>
</table>

¹ In 90 % of cases

In the Brown Swiss breed, presumably because of the small number of bulls to estimate the marker effects (only 225), the increase in accuracy was quite small. In the Jersey breed, where bull numbers where intermediate between the Holstein and the Brown Swiss, results were also intermediate. This shows the importance of using large numbers of proven bulls to get accurate estimates of marker effects. Marker effects estimated within one breed do not appear to work well in other breeds. For smaller breeds, international cooperation may be required to ensure large enough numbers of genotyped proven bulls.

For proven bulls, the use of genomic information does not increase proof accuracy by very much, with one important exception corresponding to traits with low heritability such as daughter fertility and longevity (Table 7).

Table 7: North American project: reliability of proven bulls

<table>
<thead>
<tr>
<th>Trait</th>
<th>Proof (PTA)</th>
<th>Combined (GPTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk yield</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td>Fat yield</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td>Protein yield</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td>Productive life (PL)</td>
<td>67</td>
<td>72</td>
</tr>
<tr>
<td>SCS</td>
<td>74</td>
<td>79</td>
</tr>
<tr>
<td>Fertility (DPR)</td>
<td>62</td>
<td>69</td>
</tr>
</tbody>
</table>

Even for these later traits, the increase is not as large as for young bulls or heifers. This is to be expected: since a bull’s proof already reflects the effect of its genes, the genomic information does not add as much. The increase in accuracy due to genomic selection decreases with the amount of information already available for any animal (Figure 2).
Validation is essential

It is important to understand that not all genomic evaluations that will become available from various countries or companies in future will have the same predictive value. Their predictive value will depend upon the specific breed or population, the number of bull proofs used to estimate the markers effects, the statistical models and procedures used to derive genomic evaluation, etc.

The reliability of genomic evaluations should indicate their predictive value. However, these reliabilities will only be comparable if they are based on similar validation approaches and are calculated with similar methods. Before any genomic evaluations are exchanged between countries or breeding companies, international standards will have to be developed on what constitutes an appropriate validation, and on how published reliabilities can be derived to match validation results. This is an area where Interbull could play a useful role.

Genetic Evaluation

The Holstein results clearly show that DNA profiles can increase accuracy of evaluation, particularly for young bulls and heifers, and to some extent for cows. Given that the cost of genotyping a young bull with the 60,000 marker panel is now US $210-230, it is a “no brainer” for an AI organization to genotype all young bulls in order to increase their chances of being returned to service before investing US $40,000 to US $50,000 to progeny test them. This justifies the wide-scale use of genomic selection by AI organizations in Canada and the US. Elite breeders are also interested in using the technology to detect top cows that could become future bull dams. In Canada and the US, the Holstein Associations are offering a genotyping service for their members. Already, many animals have been genotyped with the 60,000 marker panel (Table 8). While the cost of this panel may still be high for the average producer, smaller, much less expensive panels in the US $20 to US $50 range are likely to be available in future, which will be less accurate than larger panels but will permit the screening of heifers to decide which ones to keep for herd replacements.
The USDA and the Canadian Dairy Network (CDN) have started publishing official genomic evaluations this year, which replace traditional evaluations. Genomic evaluations of females are public as soon as computed. For males, evaluations are public only once the calf has been enrolled into an artificial insemination program or has reached two years of age.

Genomic evaluations have been calculated every two months but may be calculated monthly in the future. Genomic information is currently propagated to non-genotyped progeny. Methods are being studied to propagate it also from progeny to parents, so that the evaluation of a cow is affected by the genomic information of its daughters or sons. The marker effects will be recalculated at a minimum three times per year, with each official national evaluation.

The transition to the use of genomics is fairly transparent for producers. In Canada, genomic evaluations are called GPA (genomic parent average) or GEBV (genomic estimated breeding value) rather than PA or EBV to indicate they contain genomic data. The main difference with current evaluations is the increased reliability resulting from the use of the animal’s DNA profile, and eventually of the DNA profiles of its relatives. The letter G has been added to the information listed for each animal to indicate whether or not it was genotyped. This applies to cows as well as bulls.

Genomic selection could in future be applied to embryos. Essentially, a large number of embryos could be produced from the best females in the breed. A biopsy would then be obtained from each embryo before it is frozen in order to determine its genotype, then only embryos with the best genomic evaluations based on their DNA profiles would be implanted. This would increase selection intensity and therefore genetic progress, and save the cost of implanting embryos and raising calves that are later unwanted based on their genomic data. The amount of DNA in an embryo biopsy is very small (about 30 picograms), and while sufficient for characterization of a few specific genes through PCR, it is currently not enough for genotyping with the 60,000 Illumina SNP panel. However, given the current rapid advances in whole genome amplification (WGA), there is a good chance that this problem will be resolved in the not-too-distant future.
Impact on selection schemes

Genomic evaluations are much less accurate than proofs. However, they can be obtained much earlier in the life of the animal. This makes it possible to reduce the interval between generations considerably. The reduction in generation interval more than compensates for the lower accuracy of GEBV so that, if young animals with genomic evaluations are used as parents of future sons and cows, the annual rate of genetic progress for the traits selected currently can increase by 60% or more (Table 9). This is particularly important for low heritability traits such as fertility, longevity and health, where traditional selection is not as effective.

Table 9: Genetic progress for 3 selection schemes when GEBV reliability is 60 %

<table>
<thead>
<tr>
<th>Selection scheme</th>
<th>“Proof” REL</th>
<th>Sire-son interval (years)</th>
<th>LPI points per year</th>
<th>% more progress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progeny testing only</td>
<td>90 %</td>
<td>5.5</td>
<td>171</td>
<td>0</td>
</tr>
<tr>
<td>Pre-selection of young bulls on GEBVs, then progeny testing</td>
<td>90 %</td>
<td>5.5</td>
<td>187</td>
<td>10</td>
</tr>
<tr>
<td>Genotyped young bulls used as sires of bulls and cows</td>
<td>60 %</td>
<td>1.8</td>
<td>272</td>
<td>59</td>
</tr>
</tbody>
</table>

Based on these results, one could conclude that progeny testing of bulls is no longer required. However, there are both practical and theoretical reasons for a minimum amount of progeny testing. First, the accuracy of genomic predictions could decrease over time unless there are enough new progeny tested bulls each year to re-estimate the marker effects. Some bulls with semen marketed on the basis of their genomic evaluation will get an accurate “commercial” proof, but there may not be enough of them to do this effectively. Essentially, evaluations from proven bulls are the “fuel” that allows the genomic selection machine to keep going, and we cannot afford to run out of this fuel. Second, bulls marketed on the basis of their genomic evaluation should also be tested for semen fertility and, if they are used on heifers, for calving ease, to avoid problems in the herds of producers that use these bulls. Finally, one must recognize that genomic selection is in its infancy, and that there are still risks attached to it. It has taken decades to build the efficient progeny testing programs we have today, we know they work, and it would be extremely difficult to build them back up should their size be reduced excessively. Therefore, a minimum of caution is warranted. Nevertheless, strategies will vary for each market and company. In New Zealand, LIC announced it would cut progeny testing from 300 to 100-150 bulls, and put the major emphasis on “DNA bull teams”. CRV has announced a reduction of 260 to 200 progeny tested bulls. In North America, the approach may vary substantially from one AI organization to the next.

Current selection schemes have increased the rate of inbreeding in most dairy breeds. One may wonder if genomic selection will improve the situation. With genomic selection, we rely less on the parent averages of animals in order to select them, and therefore are more likely to find new sources of elite genetics, which should reduce inbreeding per generation. However, the shorter generation interval in genomic selection schemes could reduce or eliminate this advantage, so that it will be prudent for the industry to be vigilant about increases in inbreeding. The good news is that genomic selection will provide better tools to examine the effect of inbreeding. With the large marker panels that are currently being used, it becomes possible to measure the degree of homozygocity of populations, and to see which regions of the chromosomes of an animal are identical by descent. The challenge will be to ensure that the new tools are used wisely in selection programs.
Three products on the semen market

With the advent of genomic selection, producers that participate in young sire testing programs will benefit from a higher quality product, since all young bulls will have been genotyped and will have been selected by AI companies on the basis of their GPA (parent average plus genomic profile). This GPA will be more accurate than the parent average that was used before to select young bulls.

In addition, most AI companies will likely offer groups of bulls with high GPAs for specific selection objectives. The reason for offering bulls in groups is to decrease the risk associated with the lower accuracy of genomic evaluations compared to proofs. While the ultimate proof of an individual bull may vary quite a bit from its initial GPA, the average proof of a group of bulls is likely to remain closer to their average GPA. Such bull teams are well suited to clients that are less concerned about their cows’ individual sires, such as large commercial herds. The bulls are three years ahead of proven bulls on average, so their genetic merit should be higher on average, which means more genetic progress for the herds that use them. Semex has already begun to offer this type of product with Genomax bull teams. Some teams are geared to large commercial herds, with emphasis on cow longevity (herd life or productive life), daughter and bull fertility as well as good production and functional conformation, while other teams are closer to the balanced breeding approach.

AI companies will continue to offer semen from proven bulls. This remains the safest bet for producers who are concerned about the individual sires of their cows. The cost to produce progeny tested bulls is higher than for genomically evaluated bulls, but the demand is likely to continue unless genomic selection becomes a lot more accurate than it is today.

Conclusions

Genomic selection is probably the most exciting development in dairy cattle breeding since the advent of frozen semen. It is already becoming an integral part of dairy cattle selection in North America, and this is likely to occur all over the world.

Genomic selection has nothing to do with genetically modified organisms (GMO), where genes from one species are introduced into another. Instead, it uses the increasing knowledge of the bovine genome to permit more effective selection. It should lead to a faster rate of progress for all traits, especially those with low heritability such as fertility, longevity and health, which were difficult to improve with traditional selection methods. In the end, it should bring more profit to the dairy producer.

Genomic selection does not mean the end of record keeping and genetic evaluations. On the contrary, good data and good evaluations are essential for genomic selection to keep working.

The new approach is in its infancy currently, which means there is considerable room for future improvements. These include the use of more effective statistical methods, the discovery of new genes and mutations that could be integrated in future genotyping panels, and the development of smaller panels with much lower cost for screening of selection candidates by breeding organizations and producers.

Although the dairy industry has made a big leap forward in the use of genomic selection, thanks to the availability of accurate estimates of the genetic value for proven bulls, and of frozen semen from which to extract DNA, many other livestock or plant breeding industries are likely to follow suit.
GENOMIC POTENTIAL FROM BOVINE EMBRYO BIOPSIES

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Introduction

Spectacular genetic improvements have been achieved in agriculture in the past decades with traditional selection methods, especially in cattle. Genetic potential of some herds is such that nowadays, the sales of high genetic value animals or embryos represent important revenues for many breeders. For this reason, these master breeders are always receptive to novel approaches to improve the genetic quality of their animals. The advancements achieved in the last years in embryology and molecular biology could bring these breeders to the next level in terms of selection of their best animals. Until now, selection of the most promising subjects has always been done after calving but technology today has the potential to bring this to the embryonic level. This would allow breeders to do a pre-selection of their embryos before transfer and only keep the embryos with the better potential and stop wasting recipients with embryos of lesser genetic potential.

Useful information enclosed in embryonic cells

Two different types of nucleic acids are found in mammalian cells: DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). Both will give you completely different information about the cells studied. The DNA found in an embryo result from the combination of half of the genome from each parent that conceived this embryo. Genomic DNA is static and will never change from the development of the embryo until the death of the adult animal. The genomic DNA represents the animal’s genetic make-up and therefore any information that can be collected from its make-up represents the animal’s future potential. On the other hand, RNA is a very dynamic type of nucleic acid that acts as a messenger to transmit the message enclosed in genomic DNA via the production of the proteins responsible for proper embryonic activity. Therefore, RNA will provide information about the immediate status of the embryo. The RNA levels vary tremendously when cells are exposed to different environments or conditions. For that reason, the RNA content of an embryo could give us critical information on the quality status of the embryo and its ability to resist to freezing or its potential to induce a pregnancy (Figure 1).
Modern sexing procedure developed at LAB

For many years now, the major genomic information gathered from DNA extracted from embryo biopsies was the sex of the embryo. Still today, we are far from the 100% accuracy hoped by the breeders and veterinarians. Published reports and our own experiences at LAB with commercial sexing kits revealed inconsistent and limited results in terms of efficiency and accuracy (Garcia et al., 2001, Hasler et al., 2002, Lopes et al., 2002 and Yu et al., 2007). For these reasons, LAB decided to develop its own embryo biopsy sexing technique. This new method had to be faster, friendly-user, more effective and more accurate than commercial kits available at this time. The final objective was to provide a very effective and precise sexing service to breeders.

LAB’s sexing method relies on the use of real-time PCR which is a more sensitive technique when compared to conventional PCR. It is also possible to monitor the embryo biopsy quality and the amount of DNA present in the tube by following in real-time the increase of fluorescence during the amplification step. It is possible to amplify a gene fragment from the X chromosome as an internal control and another fragment from the Y chromosome to discriminate male embryos. Both fragments are amplified with the same exact efficiency and thus, no bias is introduced by over-amplification of one fragment versus the other which could result in a wrong diagnostic. Sex diagnostic is easily done in a few minutes by computer analysis after the completion of the amplification run which takes about one hour (Figure 2).
Figure 2. Results obtained from a female and a male embryo after the sexing procedure.

So far, out of 1385 embryos biopsied and sexed with this method, only 12 were undetermined which gives an efficiency of 99.1%. After modification of certain components and procedures, out of 626 sexed biopsies, we had an efficiency of diagnostic of 100%. Moreover, our technique is 100% accurate which means that the sex of all embryos sexed and transferred at LAB were confirmed by ultrasound at day 60 and were in accordance with the diagnostic given by the technique.

Other diagnostic tests currently developed at LAB

The second diagnostic test developed was the identification of embryos carriers of the red factor mutation that results in the red coat color of Holstein cows. Since there is a recent interest for red and white Holsteins, development of such a test would permit breeders to accelerate the selection of red animals. This is especially interesting when two black and white Holstein animals carrier of the red factor mutation are used for embryo production since only 25% of the progeny will be red and white. Currently a trial period is underway to validate the efficiency and accuracy of this new test but similar results than those obtained for the sexing technique are expected (Figure 3).

Figure 3. Red factor identification in different embryos

Essentially, all phenotypic aspects resulting from a mutation in a single gene could be identified in biopsies from embryos with this technology with accuracy close to 100%. As an example, many genetic diseases or traits could be identified like BLAD, CVM, Curly Calf or Polled gene.
Genotyping of embryos with the Bovine SNP50 Beadchip

Another tool breeders could find very useful is the possibility to get the genomic evaluation using the Bovine SNP50 Beadchip at the embryonic level. This DNA chip has been developed by a North American consortium and contains more than 50,000 single nucleotide polymorphisms (SNPs) that can be correlated to different phenotypes and characteristics of the adult animal. However, since we are starting with a very limited amount of cells when using embryo biopsies, the main challenge is to amplify the whole genomic DNA present in the biopsy to have enough material for hybridization on the SNP50 Beadchip. The most powerful commercial methods available at the moment recommend to start with a minimum of more than 1000 cells. This is far greater than what is found in embryo biopsies where 10-12 cells are generally isolated. Moreover, the accuracy of these kits to cover the entire genome starting with a few cells (Handyside et al., 2004, Jiang et al., 2005, Park et al., 2005, Peng et al., 2007 and Lee et al., 2008) or even bovine embryo biopsies (Le Bourdhis et al., 2009) without losing any regions varies considerably. Such a low accuracy is unacceptable especially if breeders decide to transfer an embryo or not based on its genomic evaluation. Fortunately, the kits and techniques available on the market have a lot of room for improvement to upgrade their efficiency and accuracy as much as possible (>98%), even when starting with only 10 cells from embryo biopsies. Scientists will have to look in more details at this aspect if ever producers would like to apply genomics to embryo biopsies.

Quality status of the embryo

DNA alone does not give any pertinent information on the quality status of the embryo. Today, embryo quality is based on morphological criteria (International Embryo Transfer Society). Embryo conditions like the metabolic state, the capacity to resist to freezing or more importantly, the potential to induce a pregnancy and produce a healthy calf cannot be answered by studying genomic DNA alone. Therefore, evaluation of RNA levels of specific genes playing key roles in these physiological processes is necessary.

Preliminary studies demonstrate the possibilities of using RNA measurement of specific genes from embryo biopsies to predict pregnancy outcomes (El-Sayed et al., 2006 and Jones et al., 2008). Validation studies and further research need to be done to identify which genes are the best markers to predict the embryo quality.

Conclusion

Huge amounts of information are contained in the few cells collected from embryo biopsies. The recent advancements in molecular biology give us the possibility to collect, analyse and interpret these data. With these innovations, breeders will have the opportunity to have access to the latest genomic tools through the collaboration of their veterinarians.
References


USE OF REPRODUCTIVE TECHNOLOGIES AND EMBRYO TYPING FOR GENOMIC SELECTION IN THE BOVINE

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Introduction

During the last decades, the progresses in reproductive physiology and improvements in embryo based reproductive biotechnologies allowed to develop a rather complete “tool box” including reproductive techniques used either for commercial purposes and/or in the frame work of genetic schemes. Used alone or in combination, their development is influenced in many different ways including ethics and general acceptance, consumer demand for specific products, regulation changes. They present today various degrees of efficiency (Ponsart et al., 2004) and for most of them continuous improvements may be expected in the future.

The recent development of genomic selection induces dramatic changes in the way genetic selection schemes are conducted (Humblot et al., 2008). In the past few years, Marker Assisted Selection (MAS) was based on a limited number of micro satellite analyses for a few Quantitative Trait Loci. Selection was performed by combining this first generation of genomic information with conventional indexes issued from quantitative genetics. The progress of the knowledge of the bovine genome and of DNA analyses together with the refinement of the position of markers in relation with genes of interest allows animal breeding companies to use today (or in the very near future) sets of thousands of genetic markers to select animals. The evolution of genomic techniques will probably make available the use of the complete genome information for selection purposes in a few years.

Due to the present and expected future changes in the organisation of selection and associated needs, the interest of the various reproductive techniques used today for commercial purposes and in genetic schemes should be revisited.

The needs for genomic based selection schemes and commercial production

Artificial Insemination (AI), Multiple Ovulation and Embryo Transfers (MOETs) and/or - depending on countries - Ovum Pick Up associated with in vitro Embryo Production (OPU–IVP) are used today to generate the future sires to be widely used following selection through highly effective but very costly progeny testing programmes. The changes associated to the use of a very efficient genomic selection can be anticipated and results from the needs and the organisation of selection schemes which are already today completely different.

1. Due to its costs and to the fact that the genetic value of a given future sire is known with enough precision from genomic analyses, progeny testing is on the way to be considerably reduced or even suppressed. Questions are raised about the need to keep a common reference basis in different populations to evaluate the changes induced by genomic selection but the trend related to progeny testing looks irremediable.
2. One of the most important features for the new selection procedures will be to considerably increase the number of candidates submitted to genomic selection to maximize the chances of getting interesting individuals that will be positively evaluated for a large number of traits. The way to produce these large numbers of animals becomes critical at least in the European context. The lack of adequate young recipients is one of the most important bottlenecks. In addition, when producing these candidate animals on farm, the amount of field work in relation with embryo transfer and in vitro production will be even heavier than today and will generate high logistic costs. In addition, this process may increase the contractual cost with individual farmers especially when a very interesting candidate is found.

3. Together with this increase of the number of candidates there will be a need to optimise the exploitation of genetic variability and the candidates will have to be produced from parents of different pedigree’s (maximum of families within a breed). At the same time breeding should be organised in a way to maximize the variability of the next generation.

4. In an attempt to improve numerous traits by genomic selection the knowledge of the relationships between genome information and the phenotypic criteria will be crucial. Following first studies (Adjaye 2005, Adjaye et al., 2007, Evans et al., 2008, Ko 2004) the development of micro arrays (aiming to study specific functions or whole genome based) will help to characterise the relationships between genotype and phenotype. Phenotyping (animal models, precise criteria and methods) becomes the bottle neck to achieve this objective. As a consequence, there will be a need for research aiming to phenotype for new critical traits and/or to improve the precision of the phenotypes for existing traits (for instance to screen better for production and reproductive traits).

5. A strong limitation for a wide use of genomic selection, out of breeding organisations /companies that will generate such systems, is the need for expert systems able to interpret the results issued from several thousands to x100 thousands of markers and the question is raised of how access to this information will be given.

In this context, AI alone, looks insufficient to generate enough animals in a given period of time and the efficiency of MOETs and OPU-IVP looks more and more critical to produce these large number of animals to be genotyped. Embryo sexing can be used at a very low cost during the process of genomic analyses. At the same time, sperm sexing can help to limit the number of embryos to be produced for this purpose and may be used in combination with in vitro fertilisation – in vitro production (IVF-IVP) procedures to avoid the present limitations of this technique in selection schemes due to the high number of sperm that are discarded and individual ability for bull semen when sex sorted by flow cytometry.

When considering the need to maximize genetic variability and due to strong limitations in reproductive efficiency, cloning do not appear today as an interesting tool in the frame work of selection schemes. However, beside selection schemes driven by breeding associations / companies, individual farmers that may get access to genomic selection, may be interested in the duplication of their best animals with the help of cloning for commercial purposes in the countries allowing the use of this process. Some applications may result from the use of transgenesis associated to cloning, however such technical options especially in the EU context, will face strong limitations in relation to ethics that will probably limit their use to types of production different from agronomics.

All these trends may lead breeding associations / companies to completely control the production of genomic selected animals. This will probably reinforce the use of embryo based reproductive techniques MOETs and IVP applied in station to give birth of previously (pre) selected animals within a given structure / company and not on farm. In this context, the success of embryo typing before transfer may be more and more critical for the breeding organisations.
Why Embryo Typing?

The interest of embryo typing has been discussed before the emergence of the new techniques for genomic selection that includes thousands of markers. As soon as MAS based on a limited number of microsatellites could be used, advantages were found due to its potential interest for screening the embryo for several traits at the same time. Doing the typing and selection early in life was expected to be a solution to shorten the generation interval and to limit the costs for producing the high number of calves and the cost of subsequent progeny testing that were needed to achieve multi character selection. Today the potential advantages of combining embryo production and genotyping are even higher considering the needs of genomic selection based on a maximum of candidates and use of various genetic origins to preserve genetic variability. To satisfy this, due to the efficiency of “intensive” embryo based reproductive techniques it is relatively easy to increase the number of candidates by increasing the number of flushes in MOETs. The number of produced embryos by a given period of time can even be multiplied by 2 or 3 (Ponsart et al., 2004) by the use of repeated OPU-IVF sessions that present the additional advantage of increasing genetic variability if different bulls are used for different OPU sessions or even within a session. Consequently, producing more embryos is really feasible today but strong limitations exist for giving birth to a very large number of candidates that would be genotyped after birth. One of the main bottle neck that meets breeding organisations in Europe is the limited availability of female recipients. This is reinforced by the fact that the efficiency of embryo transfer is much lower when using cows instead of heifers as recipients (Ponsart et al., 2004). In addition to this, high costs will be induced by the transfer of a very large number of recipients maintained pregnant until birth and the valorisation potential of the non selected calves will be low.

For these reasons, genotyping the embryos and selecting them before transfer appears as a seducing scenario to maximize the chances to find, interesting individuals for multiple traits.

Embryo Typing results

The above described considerations led us to explore different scenarios to test the feasibility and efficiency of embryo typing. As well as for the few results reported in the literature (Peippo et al., 2007, Guignot et al., 2008) all the typing work was done initially with a limited number of markers. Peippo et al., 2007 have shown that it was possible to genotype embryonic biopsies for a limited number of microsatellites and to get subsequent pregnancies after transfer of the corresponding biopsed embryos. Similarly, Guignot et al., 2008, reported the possible use of embryo genotyping for a very limited set of markers to screen for sensitivity to scrapie combined with sex determination. A first set of results has been obtained by using the set of 45 micro satellites on which the first generation of MAS was based.

In a first step, in vitro produced embryos were used to assess the accuracy and repeatability of embryo based genotyping. In these series of experiments, Day 6 embryos were biopsed and each blastomere from the biopsy was submitted to embryonic cloning (Lebourhis et al., 1998) to reconstitute full blastocysts. On the mean 2 full blastocysts were obtained from cloning biopsy blastomeres and more than 95% of the embryos survived in culture following biopsy. The results of typing obtained from the reconstituted blastocyst and the donor embryo were subsequently compared. From a total of 41 samples, the proportion of typed samples was >90%. The typing of the cloned embryos corresponded well to the typing of the original embryos and genotypes were fully compatible with the genotypes of the parents. The error rate, when considering differences between the different types of samples was 3% and all errors were due to the lack of identification of one of the alleles (drop-out).

From a second series of experiments, the typing results between biopsies of 10-20 cells performed at the blastocyst stage and the rest of the embryo were compared. Whole Genome Amplification (WGA) was applied on cell extracts from the biopsy before typing. From 60 samples, 95% were genotyped and a similar rate of drop out was observed (2-3%).
Another set of 40 samples was used to evaluate the minimum number of cells to be biopsed before pre amplification. WGA was performed on all samples and allowed genotyping in 98% of the case with very low drop out rates from biopsies of 8-10 cells. This rate was much higher in biopsies containing less than 5 cells.

From further series, the correspondence between results of embryo typing and of typing realised in foetuses and young calves was found excellent (13 couples embryo/calf or foetus with same typing results). Those first results obtained with biopsies issued from in vivo or in vitro embryos produced in station and biopsies performed in a central laboratory were completed by a set of results were biopsies were made on farm following the collection of in vivo produced embryos (Lebourhis et al. 2008). Typing was made by using the usual set of 45 micro satellites markers which was completed by the analysis of a set of 13 Single Nucleotide Polymorphism (SNP) markers. From 57 biopsed embryos, the total detection rate was higher for SNP’s than for micro satellites (70.2% vs 31.6%; p<0.01). The detection rates of the markers were not significantly affected by embryo stage, biopsy size or sex of the embryo. However, from these series of biopsies made under farm conditions the proportions of markers detected where much lower than when the biopsies were prepared in laboratory and immediately followed by whole genome amplification suggesting that the conditions of preparation and/or transportation may affect the efficiency of the system.

Developmental ability and pregnancy rates following transfer of biopsed embryos

Additional experiments were carried out to evaluate and compare the developmental ability of biopsed embryos after in vitro culture and the pregnancy rates following transfer of in vivo produced embryos previously biopsed and frozen.

Embryo survival following the biopsy of in vitro produced embryos was not different from the rate observed for non biopsed embryos from the same series of production that were used as controls (58/64 vs 18/20; 90%). From subsequent series, the embryonic development in vitro following biopsy of in vivo and in vitro produced embryos were not different (62/70; 89% vs 41/44; 93.2%). These results indicate that the effects of the biopsy by itself on subsequent embryonic development are very limited whatever the system used.

Pregnancy rates following the transfer of fresh or frozen biopsed in vivo produced grade 1 and 2 (IETS classification) embryos were not significantly different and close to 60% when transfers were made either on farm (100/159; 62.8%; fresh vs 36/61; 59%; frozen) or in station (54/90; frozen). In addition to this, from more recent series in station, it has been shown that grade 3 embryos may be used as well as pregnancy rates following transfer of those where not different when compared to results obtained with grade 1 and 2 (Gonzalez et al., 2008).

When considering these results, the typing from biopsed in vivo produced embryos looks realistic as the development rates and pregnancy rates following transfer of biopsed and frozen embryos does not seems too much affected by the biopsy itself. In addition those results show that most of the embryos and even grade 3, could probably be kept in the process and this would allow to genotype most of them. Despite good pregnancy rates have been reported with frozen in vitro produced embryos in many countries (Ponsart et al., 2004), improvements are probably necessary for those, because of the selection usually applied before freezing and lack of references on pregnancy rates following transfer of large numbers of biopsed and frozen in vitro produced embryos.
Genetic and economical interest

Calculations have been performed to estimate the advantages of using embryo typing in association with MOETs when compared to use of conventional embryo transfer alone. In a first study, simulations were made from real series of observations obtained from females included as donors in genetic schemes and performances of their sons evaluated at various ages (Colleau et al., 2008). Those simulations based on the use of the first generation of MAS markers, showed that the advantages related to the use of embryo typing are very significant at time of early evaluations (until 1 year of age) but are not present at the time of final evaluation. This indicated that with this typing method (limited number of micro satellites) embryo typing scenario suffered from the lack of precision of the genetic information related to young mothers and to the lack of precision of genotyping evaluation which are used to select those early in life. These defaults are much less important now with the better knowledge associated to young parents due to the accumulation of genetic information within the genetic scheme and becomes completely negligible/inexistent today with the gain of precision related to the use of high density genotyping with the 54k SNP chip and in the case of Whole Genome Evaluation. Other types of simulations based on the costs induced by different scenarios to produce the same number of bulls of a same genetic value revealed that substantial gains can be raised with the help of embryo typing if the whole set of reproductive techniques is well controlled.

Additional economical and genetic simulations should be performed in this new context of using high density markers chips to precisely evaluate the costs and advantages for the genetic schemes of such procedures based on embryo typing. Limitations may be encountered in relation with the technical feasibility of using amplified DNA together with the last generation of high density markers chips. If needed, alternatives may be found by using other types of chips fully compatible with the analysis of pre amplified DNA allowing a pre screening of the embryos before performing full genotyping in calves.

Conclusion

In the present context showing very impressive improvements in the use of MAS, it is likely that the use of a set of intensive reproductive techniques together with embryo typing will bring very significant advantages to breeding organisations able to monitor all those techniques with efficiency. It is clear that the emergence of the new methods for genomic selection makes all improvements related to embryo production in vivo or in vitro and associated techniques very attractive for breeding organisations and companies willing to valorise as much as possible the advantages of genomic selection.

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References


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