

## Consequences of Mishandling Frozen Semen and Embryos

Brad Stroud, DVM  
Stroud Veterinary Embryo Services, Inc  
Weatherford, Texas

### Abstract

Biological cells like bovine sperm and embryos are frozen by exposing them to cryoprotectants then slow cooling the samples at specific rates to allow the exodus of intracellular water molecules prior to being plunged into liquid nitrogen for long term storage. Slow cooling rates along with cryoprotectants allow the formation of very small ice crystals in the extracellular solutions during freezing. The primary goal during cell freezing is to remove intracellular water, which minimizes the formation of intracellular ice that is created when intracellular water molecules crystallize at sub-freezing temperatures. Intracellular ice damages cell membranes, cellular organelles, and even chromosomes therefore intracellular water must be removed before reaching crystallizing temperatures. Bovine embryos are typically exposed to either glycerol or ethylene glycol for several minutes at room temperature and then ramped to a temperature of about  $-35^{\circ}$  Celsius (C) before being plunged into liquid nitrogen ( $-196^{\circ}$  C). It is important to note for both sperm and embryos that once they are cooled to a point below  $-130^{\circ}$  C, the glass transition temperature of water, they cannot be raised above that temperature and then be re-exposed to below  $-130^{\circ}$  C or cell damage can occur<sup>8</sup>. The damage to the cells is caused by a reorganization or transformation of very small ice crystals in the extracellular fluids into much larger crystals during the temperature changes from below  $-130^{\circ}$  C to above  $-130^{\circ}$  C, and back to below  $-130^{\circ}$  C. This biological effect is called recrystallization. Damage occurs when the transformed large crystals physically invade the cell membranes and cellular organelles of either sperm or embryos. The severity of damage to cells is dependent upon two factors; one, how high the temperature gets above  $-130^{\circ}$  C, and two, the duration of exposure above  $-130^{\circ}$  C. Rapatz<sup>8</sup> reported that although some cell damage can occur at  $-130^{\circ}$  C ice is relatively stable at  $-100^{\circ}$  C, but becomes more vulnerable at  $-80^{\circ}$  C. Since the temperature in the necks of most standard ranch Dewars ranges from to  $-75^{\circ}$  C all the way to room temperature, it is very common for frozen semen and embryos to be exposed and damaged, or even destroyed, during routine handling by those involved in daily Dewar management. The purpose of this paper is to review the damaging effects of recrystallization, and to point out common mistakes made by those who routinely handle frozen semen and embryos on the farm or ranch. Also, the economics of damaged semen and embryos due to mishandling will be discussed.

### Introduction

It is theorized by low temperature biologists that frozen semen and embryos stored properly in liquid nitrogen at  $-196^{\circ}$  C, or liquid nitrogen vapor below  $-130^{\circ}$  C, will stay viable for at least one thousand years. Cellular metabolism at those temperatures essentially ceases, therefore the cells don't age<sup>5</sup>. However, frozen semen and embryos are routinely handled by a myriad of people from the time of freezing and processing until ultimately being thawed for use in a cow. Unfortunately, most of those people, including veterinarians, who come into daily contact with frozen semen or embryos, have never been formally trained in the safe long-term storage of cells in liquid nitrogen, and exposure damage to reproductive cells during routine handling. Essentially, once commercially frozen semen and embryos are shipped from the laboratory of origin, their fate is usually in the hands of individuals untrained to handle them. At

least some exposure damage is eminent under those circumstances. Frozen semen evaluations made by professionals in the field of bovine embryo transfer indicate that improper semen handling is likely a common cause of unfertilized ova collected from bovine donor females (Stroud personal observation).

### Observations from frozen thawed semen

Schrick<sup>13</sup> reported data on more than 740 superovulated donor collections where fertilization and embryo production were correlated with frozen semen evaluation data. The semen was microscopically spot tested immediately post-thaw, but prior to donor breeding. All semen was evaluated by one technician, and the same technician inseminated the donors to eliminate variability. The idea was to recognize and eliminate poor or unacceptable quality semen at the time of donor breeding in order to minimize the number of donor collections with zero or few viable embryos, and to increase the overall percentage of viable embryos recovered from superovulated donors. Five parameters were evaluated during a spot test; 1) concentration or number of sperm, 2) percent motile sperm, 3) rate of forward movement, 4) direction of movement, and 5) morphology. An overall score of excellent, good, fair, poor, or unacceptable was assigned to each sample. Sire name, sire registration number, stud code number, and batch code number was recorded for each unit of semen thawed. As predicted frozen semen graded as overall excellent produced a higher percentage of grade one embryos than overall good quality frozen semen, good better than fair, and overall fair better than poor quality semen. See Table 1.

**Table 1**

Overall Semen Quality	% of Total Ova Grade 1 Embs	% of Total Ova Degenerate
Excellent	61.2	16.1
Good	55.7	22.7
Fair	53.9	26.5
Poor	33.7	51.7

Semen graded as unacceptable was discarded most of the time unless semen from a backup bull wasn't available. Although there were only a small number of donors inseminated with unacceptable quality semen in this commercial setting those that were produced mostly unfertilized ova. See Table 2.

**Table 2**

Overall Semen Quality	% of Total Ova Grade 1 Embs	% of Total Ova Unfertilized
Unacceptable	3.1	88.9

The data that wasn't included in this study, but was documented, was the origin of the frozen semen shipped to SVES for donor breeding. The location from which semen was shipped had a significant effect on the number of semen collection codes (batches) that were classified as unacceptable or poor. Semen samples shipped directly from bull studs where it was collected, processed, and stored had an unacceptable classification rate of only two per hundred batches evaluated. On the other hand, semen samples hand delivered or shipped from animal breeders to SVES had an unacceptable rate of eight per hundred batches examined. In other words, semen that has been in the hands of animal breeders and other sources at some point post freezing was

four times more likely to be classified as unacceptable compared to that stored and shipped directly to SVES from a bull stud. See table 3.

**Table 3 (Beef bulls only)**

<b>Origin of Shipment</b>	<b>Total # of Shipments</b>	<b># Classified Unacceptable</b>	<b>% Unacceptable</b>
Bull studs	426	9	2.1
Owners/others	314	25	8

This data strongly suggests that bull studs are doing a good job of processing, freezing, and shipping semen, but owners are guilty of mishandling it once they get it. My personal observations support that data. Having watched animal breeders handle frozen semen for twenty-six years, it's obvious that most have not been trained how to protect their samples from thermal exposures. One of the most common exposure mistakes made is during routine thawing for insemination. Canisters are routinely raised too high and for too long in the neck of standard ranch Dewars while the technician searches for the proper cane. Cane tabs are usually coded by the bull stud that processed the semen with a prefix code number for the stud followed by a breed code abbreviation then a unique number for that particular breed of bull. This can be confusing to an AI technician. Instead of having the bull's name printed on top he sees only a code number. If the technician doesn't have the cane codes recorded often he will lift a cane into ambient air and secure it above the frost line in the Dewar's neck, remove a straw from a goblet, wipe the frost off the straw, and read the printed information. If the straw being examined for data is the proper bull, the straw is dropped into a warm water bath for thawing. If not, it is placed back into the goblet from which it came and the search for the proper bull continues. The results of this action can have detrimental effects on not only the straw that was wiped free of frost, read, and replaced into the goblet, but the rest of the semen stored in that exposed canister as well. Again, the cellular damage occurs when the internal temperature of the semen straws rise above  $-130^{\circ}\text{C}$  and then are re-introduced to below those temperatures.

### **Exposure Opportunities**

There are many occasions during routine handling when frozen reproductive cells are exposed to potentially damaging temperatures. Bull semen is collected, processed, and frozen in plastic straws by highly skilled technicians at bull studs who are acutely aware of the consequences of thermally exposing them. Once frozen, the plastic straws are packaged in plastic goblets attached to aluminum canes by a technician working with the samples under liquid nitrogen or liquid nitrogen vapor very close to the liquid vapor interface. The manipulating temperature at either of these points is well below the critical glass transition temperature of water ( $-130^{\circ}\text{C}$ ). The aluminum canes are then swiftly moved to canisters in large storage Dewars where they safely reside until a request is made by the owner to ship them to a breeding facility.

When a shipping request is made, a skilled technician will properly charge a dry shipping Dewar, retrieve the requested amount of frozen semen, and quickly move it into the shipper. At no point while transferring the canes of semen from a large storage Dewar to a dry shipper will the technician allow the frozen straws to reach temperatures above  $-130^{\circ}\text{C}$ . A freshly charged dry shipper's belly temperature should be about  $-185^{\circ}\text{C}$ , which will safely maintain the structure of ice during shipping.

Once the dry shipper has left the bull stud, however, things can and often do go awry. The list of potential wrongdoers is long; ranch hands, AI technicians, cattle owners, family members of cattle owners, farm or ranch secretaries, dairy employees, couriers, veterinarians, embryologists, and even visitors to the farm are sometimes asked to handle shipped or stored frozen semen and embryos. Very few, if any, on the list have ever been trained in proper handling techniques for frozen biologicals. There simply isn't a curriculum that exists to do so. There are several times when exposure is most likely to happen. The following is a list of some of those opportunities:

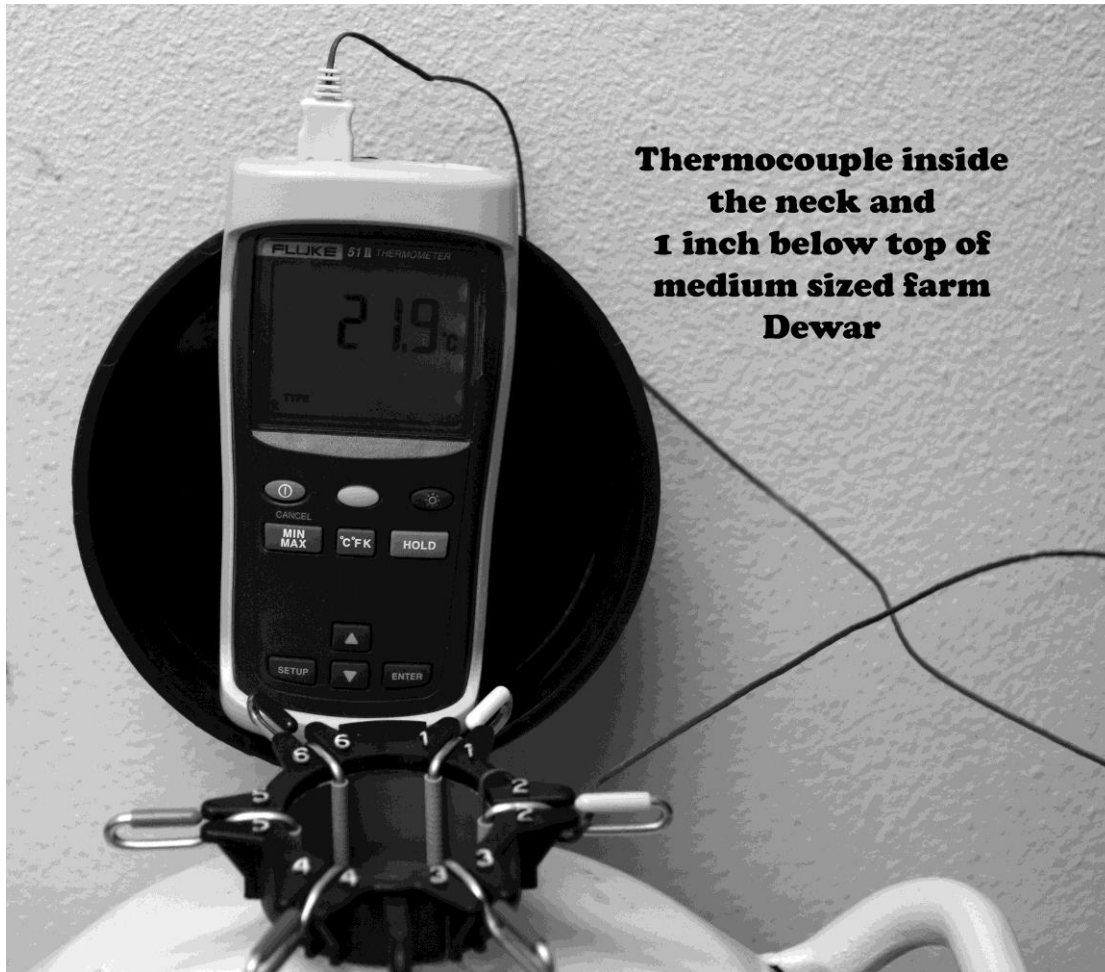
1. Receiving and transferring samples from a dry shipper to a storage Dewar
2. Thawing samples
3. Taking inventory
4. Preparing samples for shipment
5. Breaking a cane (transferring one or more straws from one cane to another)

Any or all of these events performed improperly exposes frozen semen and embryos to temperatures that can lead to cell damage or death. The damage from each exposure event is cumulative<sup>11,15,16</sup>. For example, each time that a canister containing canes of frozen semen is exposed high enough in the neck of a small or medium sized Dewar, and for ample time to allow the straw's internal temperature to rise above  $-130^{\circ}\text{C}$ , a certain amount of damage occurs to the exposed semen in that canister. If the mistake is repeated over and over, each exposure causes damage that is additive. So, poor handling habits by a technician can result in cumulative damage that can decrease the sperms fertilizing capabilities, and in some cases lead to total infertility. These mistakes often lead to long and arduous witch hunts by veterinarians, nutritionists, bull stud representatives, and others trying to diagnose the etiology of low conception rates in apparently well managed herds. In cases where semen has been mishandled, more often than not, nutritional insufficiencies and poor heat detection inappropriately get blamed for the problems. Heads are often left scratching and imaginative diagnoses are made to appease the owner.

The real problem in the field seems to be logic on behalf of uneducated handlers. If frozen liquid, like a straw of bull semen, remains solidified or crystallized during or after an exposure, the perpetrator logically thinks no harm has been done. Since the ice structure has remained intact, at least to the naked eye, he thinks the sperm should be fine. Unfortunately, that's not the case. As previously mentioned, ice becomes very unstable at temperatures above  $-80^{\circ}\text{C}$  and significant damage can occur to cell membranes and delicate cytoplasmic organelles after such an exposure.

### **Liquid Nitrogen, Vapor, and Temperature Gradients in Dewars**

The boiling point of the liquid nitrogen is  $-196^{\circ}\text{C}$ . However, the vapor phase of nitrogen can vary from  $-190^{\circ}\text{C}$  to  $-50^{\circ}\text{C}$  depending on how far from the liquid/vapor interface and how close to ambient air the measurement is taken. This is important information relative to small or medium sized farm Dewars. For a Dewar three quarters full of liquid nitrogen the temperature of vapor just above the liquid/vapor interface is usually very near or below  $-190^{\circ}\text{C}$ . Conversely, the temperature one inch from the top of the same Dewar is only a few degrees cooler than ambient temperature (figure 1).



**Thermocouple inside  
the neck and  
1 inch below top of  
medium sized farm  
Dewar**

Figure1. Temperature (Celsius) of a storage Dewar one inch below the top of the vessel

Measurements in between those landmarks vary in a gradient manner. Table 4 shows the results of plotting the temperature of a typical farm storage Dewar that is three quarters full of liquid nitrogen beginning at the top of the neck (room temperature) and dropping at one inch intervals to a point 8 inches below the top of the vessel.

<b>Table 4</b>	
<b>Depth in Neck</b>	<b>Temp °Celsius</b>
0 inches	24.1
- 1 inch	21.9
- 2 inches	11.9
- 3 inches	-23.0
- 4 inches*	-47.1
- 5 inches	-73.6
- 6 inches	-110.5
- 7 inches	-160.4
- 8 inches	-181.2
* Frostline of this Dewar	

Notice that the temperature at the frost line of this particular Dewar, which is four inches below the top of the vessel, is  $-47.1^{\circ}\text{C}$ . That's 83 degrees warmer than the safe temperature of  $-130^{\circ}\text{C}$ . Considering that many AI technicians routinely raise canisters containing canes of semen to the top of a Dewar for searching and/or thawing, this table illustrates how vulnerable samples are during those routine handling procedures. The neck of Dewars is the working area where samples are routinely handled and consequently exposed to potentially damaging temperatures. Canisters containing frozen biologicals should spend only brief periods of time there since the temperature at most levels in the neck above the frost line are well above  $-80^{\circ}\text{C}$ . It is important to note that Dewars differ in their neck diameter and in their insulation properties, so the temperature gradients differ from vessel to vessel. They are not all created equal. Also, a Dewar full enough to have liquid nitrogen in the top goblet provides more protection for samples held in the neck than one that doesn't<sup>1,7</sup>. At Stroud Veterinary Embryo Services, all storage Dewars are fully charged every four weeks, and sometimes more often during the summer breeding months. Additionally, extra Dewars without canisters are used to top off the storage vessels during periods of heavy use. These steps assure that the top goblets attached to canes of frozen semen and embryos are always liquid filled. That buys an extra few seconds of safety for canisters or canes lifted into the neck when retrieving or handling units of frozen semen. Berndston<sup>1</sup> found exposure time permitting semen temperature to reach  $-45^{\circ}\text{C}$  resulted in a dramatic irreversible decline in post-thaw motility. He measured the internal temperature of a standard 0.5 ml straw of semen using an embedded thermocouple inside a straw of semen. He showed that it only takes 10 to 15 seconds for the temperature of half ml semen straws stored in a vapor filled goblet to drop from  $-190^{\circ}\text{C}$  to  $-100^{\circ}\text{C}$  when the goblet was raised to the upper third (working area) of the neck of a Dewar. The same exposure time allowed a 0.25 ml French straw to rise to  $-75^{\circ}\text{C}$ . As a result, he recommended that a cane or a canister containing canes of frozen semen be limited to no more than five to eight seconds in the neck of a Dewar during an exposure like thawing or searching for particular tab codes. Because of the significance of Berndston's suggestion Leibo and Stroud coined the phrase "eight second rule" in their training DVD, *The Guide to Handling Frozen Semen & Embryos*<sup>14</sup>. Since the temperature varies at the frost line from Dewar to Dewar the eight second rule provides a safe working time for most handling events. However, it must be emphasized that raising canisters or canes four or more inches above the frost line or into ambient air jeopardizes some samples even when applying the eight second rule, i.e., when working with a Dewar in windy conditions, which rapidly dissipates heat from frozen semen or embryo straws. A handler should never work with frozen samples in direct wind.

### **Quarter ml plastic straws**

So far, much of the emphasis of this review has been on 0.5 ml plastic semen straws, which is the standard container for commercially frozen bovine semen in the US. However, most Canadian and European countries utilize 0.25 ml French plastic straws for packaging frozen bovine semen. Additionally, most bovine embryos world wide are frozen and packaged in 0.25 ml plastic straws. Most recently, semen sorted commercially for sex in the US is being frozen in 0.25 ml French straws<sup>a</sup>. There are reasons that the respective industries have selected the small 0.25 ml straw for packaging, and that topic will not be addressed by this paper. However, the small and more slender straw does pose a challenge to technicians in the field. Because of the much higher surface-to-volume ratio in quarter ml straws, heat dissipation occurs much faster

than with 0.5 ml straws. As a consequence, anyone handling quarter ml straws should be extremely cautious when manipulating frozen samples in the neck of storage Dewars. Embryos frozen in quarter ml straws can be in serious jeopardy from mishandling, especially if they happen to be trapped very near or against the wall of the plastic straw during freezing. Unlike sperm where several million reside in a single straw, there is usually only one embryo packaged per straw. If a frozen embryo is situated against the wall of the straw, it can be destroyed by very brief (three seconds) exposures to ambient temperature if it's placed back into liquid nitrogen post exposure<sup>b</sup>. Frozen semen packaged in quarter ml straws, on the other hand, is a little more forgiving than embryos. Twenty million sperm are not all likely to be damaged or destroyed by a single exposure unless the exposure is severe. Those sperm residing against the wall of the straw may be damaged before those residing in the inner core of the straw during a brief exposure. That fact is especially important with sex sorted semen since only  $2.1 \times 10^6$  sperm are currently packaged per quarter ml straw. That's about one tenth of the motile sperm packaged in conventional un-sexed semen straws for beef cattle. Considering that the two million sperm in the straw have been mechanically stressed during sorting<sup>3,4</sup>, it's imperative that the vast majority of those sperm are protected from heat exposure prior to being thawed and inseminated if reasonable conception rates are to be expected.

### **About dry shippers**

Dry shippers are designed to keep biologicals safely cooled during shipping. Their internal design prevents liquid nitrogen from spilling when a vessel is tipped over. The temperature of a properly charged dry shipper should be  $-185\text{ C}$  or colder in the belly of the vessel immediately after being charged. That's obviously a safe environment for shipping sperm or embryos. An absorbing sponge-like material soaks up liquid nitrogen which prevents sloshing or spilling during shipping, yet keeps the vessel only a few degrees warmer than liquid nitrogen itself. This makes couriers happier than having to deal with spilled liquid in the cabin of their vehicle while driving down the road. Typically, dry shippers used in the livestock industry for shipping small quantities of frozen semen or embryos are smaller than most storage Dewars, so caution should be exercised when a shipment arrives at a farm or ranch. Contents should be removed immediately from the dry shipper and placed in safe storage. Be aware that lifting the Styrofoam lid from a dry shipper and seeing vapor arise from the neck does not guarantee that the samples have not been exposed to dangerously warm temperatures. At some point after having been charged with liquid nitrogen, a dry shipper will begin to warm. Figure 2 shows a dry shipper 10 days post charging. The internal temperature of the Dewar has warmed from  $-190\text{ C}$  immediately post charging to  $-138.9\text{ C}$  ten days later. That's getting very close to the glass transition temperature of water, the temperature where ice becomes unstable and recrystallization begins to occur.



Figure 2. Dry shipper 10 days post charging with liquid nitrogen. Temp – 138.9° C

Plastic goblets snapped onto canes inside dry shippers will not have liquid nitrogen in them, so time in the neck of the shipper should be kept to a minimum when viewing or handling the contents. The eight second rule also applies to dry shippers. Canes of semen or embryos



should be transferred swiftly from a dry shipper to a storage vessel to prevent possible damage during movement.

### **Assessing heat damage to frozen semen**

It is difficult if not impossible on the farm to assess physical damage done to sperm by heat exposure and recrystallization other than by an unexplained decrease in conception rates following artificial insemination. Even veterinarians trained in microscopic frozen semen evaluation cannot unequivocally diagnose heat damage unless, of course, the sperm is totally dead, or there are gross differences in post thaw motility within straws of semen from identical batch codes. Without reference to other units of semen from like batches, it is unwise to assume that a bull stud processed and froze poor or unacceptable quality semen, or that the semen has been mishandled and exposed to damaging temperatures by someone post freezing. It's difficult to distinguish between the two scenarios microscopically. However, several consistent, although certainly not pathognomonic, changes can be noted when sperm is thermally insulted; 1) an increase in the percentage of spent and damaged acrosomes<sup>6,7,10,11,12</sup>, 2) a decrease in overall motility, and 3) in some cases an increase in spastic non-linear motility (personal observation). For novice frozen semen evaluators to notice suppression in the motility of frozen semen to a single heat exposure event, most batches of frozen semen must be exposed for about 30 seconds or more to temperatures very high in the neck of a Dewar, or to ambient air, then returned to liquid nitrogen storage conditions before being thawed for evaluation. Usually 0.5 ml frozen semen straws must be exposed for about 50 seconds or more before motility totally ceases. Again, there is some variability between bulls in the amount of exposure time necessary to cause 100% loss of motility. However, acrosome damage is seen well before noticeable changes in motility<sup>1,7,11,12</sup>

Pace<sup>6</sup> reported that the percentage of intact acrosomes decreases significantly ( $P < .05$ ) in field stored sperm compared to centrally stored sperm of the same collection codes. The field stored sperm were stored in working ranch Dewars where samples were removed on a daily basis for breeding purposes. After six months of retrieving straws for breeding, it was determined that the undisturbed centrally stored semen had a higher percentage of intact acrosomes as compared to the field stored sperm that was routinely exposed to the neck of a working Dewar. In addition to visual changes in thermally challenged sperm there are some biochemical changes that take place<sup>2</sup>. However, diagnosing that kind of damage can't be done at cow side and requires money and time to do so.

At Stroud Veterinary Embryo Services semen samples microscopically diagnosed as poor or unacceptable are videotaped. About half the time, either bull owners or bull studs that froze the samples are unhappy with the diagnosis. A videotape of the exam is sent to the bull owner and/or bull stud which reveals the truth about the semen. Before sending the videotape to the concerned parties, some editing is done to help the viewers who may not be able to appreciate excellent from poor quality frozen semen without an opposite reference. In the edited video the poor quality semen is compared side-by-side with an excellent standard, which shows a substantial difference between the two. Apologies are often promptly made to the diagnostician. A confrontation between owner and stud usually takes place. Sometimes the bull stud apologizes, but most often their post thaw assessment reveals acceptable quality semen from the same batch. On many occasions I receive a replacement shipment with an identical batch code that tests satisfactorily. That's pretty good evidence of post freezing thermal exposure damage. Couple that with the fact that shipments of frozen semen shipped directly to SVES from bull

studs is four times less likely to be culled as compared to owner delivered frozen semen, and a very strong case for mishandled frozen semen by untrained personnel in the field can be built.

### **Economics of mishandled frozen semen**

Thirty years of embryo transfer has made bull studs more aware of processing and freezing better quality semen. Unlike pregnancy and calving rate data that is slow to collect with traditional AI programs, embryo collection data quickly reveals how fertile a particular bull or batch of semen is. With more at stake, semen used for donor cow insemination is more likely to be microscopically evaluated than semen used for inseminating single ovulating females. The most recent data from the AETA statistics committee report shows that 48% of dairy ova and 46% of beef ova collected from superovulated donor females are unfertilized or degenerated (cleaved but dead). Data on 746 donor collections at SVES showed that poor quality semen produced only 33.7% viable embryos compared to the AETA average of 55%<sup>c</sup>. By eliminating most of the unacceptable and much of the poor quality semen at the time of donor breeding at SVES, the percent of viable embryos was 64% compared to the US industry average of 55%. With two per hundred semen samples shipped directly from bull studs being rejected at thawing/donor insemination, and with eight per hundred samples hand delivered or shipped directly from clients being rejected, one can calculate an estimated industry loss in revenue from mishandled frozen semen. This calculation may not be perfect, but it hints at a major problem that both the AI and ET industries face that can be eliminated with recognition and client education.

Model for estimate of economic loss due to mishandled frozen semen

- 1) Consider percent viable embryos collected in 2005 was 55% of total ova collected (beef and dairy combined AETA 2005 stats)
- 2) Consider SVES managed donors (poor and unacceptable semen eliminated) percent viable embryos for 2005 were 63.9% of total ova collected.
- 3) Estimated loss of embryos due to poor semen quality can be calculated
  - a. 64% viable embryos (SVES inseminated donors)
  - b. minus 55% viable embryos (AETA industry stats/non-evaluated frozen semen)
  - c. = 9% ova wastage due to microscopically diagnosed poor quality semen. This also correlates closely with the percentage of batches of semen culled immediately post thaw. (8% shipped from owners plus 2% shipped from bull studs)
  - d. Also consider the following:
    - i. In 2005, 48,233 stimulated donors were collected (reported beef plus dairy donors combined AETA data).
    - ii. 305,319 viable embryos were collected from 555,125 total ova (55% of total ova collected AETA data)
    - iii. 45% of ova collected were unfertilized or degenerate
    - iv.  $555,125 \times 9\%$  (increase in embryo production rate at SVES due to semen culling post thaw) = 49,961 embryos lost due to poor quality semen that could have been prevented with microscopic evaluation.
    - v. Total loss to ET practitioners is  $50,000 \text{ embryos} \times \$50 = \$2,500,000$  loss.
    - vi. Consider only 50% (instead of 75%) of the loss due to mishandled semen = \$1,250,000 in lost revenue for AETA members.

- vii. 134 ETBs reporting data for AETA = \$9300 lost revenue per ETB.
- viii. Loss to clients at \$300 per embryo x 50,000 embryos = \$15,000,000 /2 = \$7,500,000.

It's highly likely that this economic model is lenient on the estimation of damage due to mishandled frozen semen. It takes into account only frozen semen that has been culled due to gross mishandling. As already mentioned, many semen samples have to be exposed for long periods of time to negatively influence motility. If consideration was given to infertility due to damaged sperm membranes as a result of mishandling that goes unobserved microscopically, the economic losses could easily be double or triple of that above. Are 45% of total ova collected from superovulated females inherently incapable of becoming fertilized and developing into viable embryos?

## **Conclusion**

Applied reproductive technologies like AI and embryo transfer have developed into huge global industries due to the ability to cryopreserve male gametes and embryos and subsequently thaw them with predictable results. However, many of those directly involved in implementing these technologies have, at some time, experienced poor, if not disastrous, results post thaw. Based upon careful statistical analysis of fertilization rates involving embryo transfer and semen evaluation, along with observations in the field by professionals trained in handling cryopreserved and stored samples, it's evident that mistakes made during routine handling can expose cryopreserved sperm and embryos to thermal insults that can damage or even destroy them.

It's normal for professionals in the AI and embryo transfer industries to look forward to emerging biotechnologies so their clients can take advantage of them. No veterinarian or animal scientist wants to be left behind. However, it behooves practitioners in the field to train animal breeders and their staff in basic handling techniques involving frozen semen and embryos, no matter what the species. It's imperative that fundamental handling techniques be employed in the field else the technologies will fail.

It's often very difficult to re-train experienced AI technicians who have established bad handling habits when thawing frozen semen, taking inventory, or moving canes of semen from vessel to vessel. The easy excuse for them when confronted with low conception rates is to blame the bull, the bull stud, or the cow but seldom their AI skills or semen handling techniques. After all, they've been handling frozen semen for years and not had any problems with damaged semen due to mishandling, at least that's been diagnosed. Also, AI schools have not thoroughly addressed the issue of proper handling techniques. I quiz my clients or their staff members who attend AI school about the issue of thawing and handling procedures. Most of them say the subject was either not addressed or given very little attention. In any case, education is the key to preventing exposure damage to semen and embryos. It's probably safe to say that every embryo transfer practitioner in the industry has been victimized by poorly handled frozen semen. How many donor collections are performed where all ova are unfertilized due to mishandled frozen semen? What about donor collections that have more than 50% of ova unfertilized or degenerated? Also, is it possible that one donor cow could handle thermally stressed sperm better than another, which would make a diagnosis of heat exposed semen difficult?

Perhaps more research is needed in this area to specifically point out damage done to sperm and embryos during brief or extended thermal exposures. Maybe ultra structural analysis of sperm membranes using scanning electron microscopy could pin point specific physical damage to

individual sperm and help determine under what conditions sperm undergoes significant changes after an exposure. Additionally, maybe a temperature specific dye could be added to semen samples that would react if the internal temperature of a straw of frozen semen warmed to above - 130° C then was re-exposed to liquid nitrogen. If a cane or even a canister of frozen semen turned up bright purple after being thawed, the technician would instantly know the sperm has been exposed to potentially damaging temperatures.

In any case, the value of bovine genetics is ever increasing due to the utilization of existing technologies like AI and embryo transfer. With new biotechnologies looming in the forefront like genetic markers and others, the value of superior genetics will only increase in value. But even with commercial dairies where semen costs are affordable, the expense of low conception rates can be devastating to the bottom line. Proper handling techniques for frozen semen and embryos are easy to implement and should be as fundamental as heat detection or passing an AI gun through a cervix. Implementing safe handling procedures for cryopreserved reproductive cells will increase the efficiency of applied reproductive biologies.

#### Footnotes

- a. Sexing Technologies, Navasota, Texas
- b. Stanley Leibo, personal communications, 2005
- c. Unpublished data, 2005 AETA statistics committee report

#### References

1. Berndtson WE, Pickett BW, Rugg CD: Procedures for field handling of bovine semen in plastic straws. *Proc of the Sixth Technical Conf on AI and Reproduction*: PP 51-58, NAAB, 1976.
2. Graham EF, Pace MM: Some biochemical changes in spermatozoa due to freezing. *Cryobiology*: 4:75, 1967.
3. Johnson LA, Welch GR: Sex preselection: High speed cytometric sorting of x and y sperm for maximum efficiency. *Therio*: 1999;52:1323-1341.
4. Maxwell WMC, Johnson LA: Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. *Molec Reprod Dev*: 1997;46:408-412.
5. Meryman HT: Mechanics of freezing living cells and tissues. *Science* 124:515.
6. Pace MM, Peterson LL, VanDellen GA, Waterman EV, Robbins RK, Sullivan JJ: Effect of field working storage upon the quality of frozen bull spermatozoa packaged in 0.5 ml ampoules and 0.5 ml French straws. *Am Soc Sci, 69<sup>th</sup> Annual Meeting*. P 194, 1977.
7. Pickett BW: Factors affecting the utilization of frozen bovine semen for maximum reproductive efficiency. *AI Digest*:19(2):8, 1971
8. Rapatz GL: What happens when semen is frozen? *Proc 1<sup>st</sup> Tech Conf on Artif Insem and Reprod*: P45 NAAB, 1966.
9. Robbins RK, Saacke RG, Chandler PT: Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. *J Anim Sci*: 42:145, 1976.
10. Rowson LEA: The storage of bull semen at low temperatures. *Vet Rec*: 65:559, 1953.
11. Saacke RG, Lineweaver JA, Aalseth EP: Procedures for handling frozen semen. *Proc of the 12<sup>th</sup> Conf on AI of Beef Cattle*: 46-61, NAAB, 1978.

12. Saacke RG, White JM: Semen quality tests and their relationship to fertility. *Proc 4<sup>th</sup> Tech Conf Artif Insem and Reprod*: P22, NAAB, 1972.
13. Schrick FN, Saxton FM, Stroud BK: Assessment of semen quality for predicting recovery of viable embryos of superovulated cattle: *Joint Conv Proc CETA/AETA*, 2003.
14. Stroud BK, Leibo S: *The Guide to Handling Frozen Semen & Embryos*: DVD, [www.biotechproductions.com](http://www.biotechproductions.com) , 2006.
15. Sullivan JJ: Sperm numbers required for optimum breeding efficiency in cattle. *Proc 3<sup>rd</sup> Tech Conf Artif Insem and Reprod*: P36, NAAB, 1970.
16. Sullivan JJ, Mixner JP: Effects of storage temperature and length of storage time upon the post-thawing motility and metabolic activity of frozen bull semen. *J Dairy Sci*: 46:850, 1963.