

The Integrity of Frozen Spermatozoa

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TECHNIQUES FOR THE CRYOPRESERVATION AND FIELD HANDLING OF BOVINE SPERMATOZOA*

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INTRODUCTION

Smith and Polge⁸⁷ first reported that bovine spermatozoa were capable of withstanding freezing in the presence of glycerol in 1950. Since then the art and science of the cryopreservation of bovine spermatozoa has continued to develop, and approximately 9 million cattle are now inseminated with frozen semen annually in the United States. The application of techniques for the cryopreservation of bovine spermatozoa to spermatozoa of other mammals has produced variable and often disappointing results. Nonetheless, the wealth of research and field experience with frozen bovine semen has led to the identification of numerous essential factors and potential pitfalls associated with the use of frozen semen. The technical information that follows is based primarily upon research with bovine spermatozoa. Although specific procedures for the freezing and handling of spermatozoa from other species, including the human, are likely to differ, many of these same factors may be equally important. Thus, this discussion is intended primarily to point out the many potentially important factors that warrant critical, systematic evaluation during the development of successful techniques for the cryopreservation of human spermatozoa. Many of these factors are discussed elsewhere in greater detail.^{14,70}

*Portions of this manuscript have been taken from "Techniques for Processing and Packaging Bovine Semen" by B. W. Pickett, W. E. Berndtson, and J. J. Sullivan⁷⁰ and "Procedures for Field Handling of Bovine Semen in Plastic Straws" by W. E. Berndtson, B. W. Pickett, and C. D. Rugg.¹⁴

TECHNIQUES FOR MEASURING FERTILITY

Much of the progress in improving the survival and fertility of frozen bovine spermatozoa resulted from laboratory studies in which post-thaw motility was used to assess treatment effects. Although motility is positively correlated with the fertility of frozen bovine spermatozoa, spermatozoa from several species, such as the dog and stallion, frequently exhibit very high motility after freezing, yet are infertile. Even for the bull, there is no completely reliable laboratory criterion for predicting fertility, and several different laboratory tests are often applied. However, each factor under study must ultimately be evaluated by actual fertility testing.

Reliable fertility data are difficult to obtain even with livestock animals. For example, one of the most widely adopted criteria for measuring fertility is the nonreturn rate, i.e., the percentage of cows inseminated that was not reported for an additional insemination in a specific period. However, such data include many inaccuracies. For example, many cows that fail to become pregnant are not reported for an additional insemination because they are sold or die, are bred by natural mating, or are artificially inseminated with semen supplied by other artificial insemination (AI) organizations. Thus, nonreturn rates frequently are much greater than actual fertility. Problems of this nature must be considered when evaluating fertility data, and efforts should be made to control those factors that can influence the reliability of the data.

PACKAGING SYSTEMS

A variety of methods for packaging bovine semen for freezing have been developed. The primary considerations in the selection of a system for packaging semen are fertility, inseminator preference, ease of handling, ease of identification, freedom from contamination, economics of storage, and the proportion of ejaculates that can be frozen. Of these the most important objective should be to obtain *maximum reproductive efficiency*. Since the various packaging systems were developed under different conditions, philosophies, areas of the world, etc., direct comparisons are difficult. In fact, it must be understood that systems of packaging, rather than packages *per se*, were being compared in most studies.

Fertility

Pickett *et al.*⁷⁰ recently summarized the results of 17 studies in which the fertility of semen frozen in ampules or straws was compared. The results are presented in Table 1. Studies shown above the broken line are ones in which fertility was greater for semen frozen in ampules. In most studies, differences in fertility due to method of packaging were small. Furthermore, the observation that neither method of packaging was consistently superior to the other is consistent with the hypothesis that some factor(s) other than method of packaging *per se* also contributed to the differences in fertility observed.

Sire \times packaging-method interactions have also been observed. The results of one study are presented in Table 2. In Herd 1, fertility averaged 70.1 vs. 49.5 percent for semen frozen in straws vs. ampules, respec-

TABLE 1 Effect of Packaging Bovine Semen in Straws or Ampules on Fertility

Packaging System			
Straws	Ampules	Difference	Reference
73.2	54.3	+18.9	61
63.7	57.9	+ 5.8	60
72.0	67.8	+ 4.2	2
71.0	67.0	+ 4.0	76
67.2	64.3	+ 2.9	5
70.4	67.7	+ 2.7	48
74.9	72.8	+ 2.1	42
71.1	69.5	+ 1.6	3
69.0	67.5	+ 1.5	46
71.5	70.3	+ 1.2	76
74.3	73.2	+ 1.1	76
61.1	65.5	- 4.4	38
70.7	73.8	- 3.1	4
70.7	72.6	- 1.9	8
67.7	69.1	- 1.4	30
73.0	73.9	- 0.9	1
67.5	67.6	- 0.1	38

SOURCE: Adapted from Pickett *et al.*⁷⁰

TABLE 2 Percent Pregnant^a After a 38-(Herd 1) and a 44-(Herd 2) Day Breeding Season

Bull	Packaging System			
	Straws		Ampules	
	No. Bred	Pregnant ^b (%)	No. Bred	Pregnant ^b (%)
<i>Herd 1</i>				
A	75	76.7	72	72.3
B	75	63.3	70	47.9
C	72	61.3	65	12.6
D	72	79.2	73	65.2
Mean	294	70.1	280	49.5
<i>Herd 2</i>				
C	101	82.6	103	66.8

SOURCE: Adapted from Mortimer.⁶¹^aLeast squares mean.^bDetermined by rectal palpation.

tively. However, the actual differences in pregnancy rates for the four sires used were 4.4, 15.4, 48.7, and 14.0 percentage points. Thus, method of packaging was relatively unimportant for semen of some bulls, but resulted in a marked difference in fertility for others. Methods of packaging should probably be selected on an individual-bull basis to ensure optimal fertility.

The largest difference between the two methods of packaging in Herd 1 was for Bull C (48.7 percentage points). The corresponding difference for the same bull used in Herd 2 was only 15.8 percentage points. Since different ejaculates were used in each herd, and because the cattle were maintained under different levels of management, a direct comparison of the fertility results

would not be appropriate. However, all ampules and straws used in Herd 1 contained 20×10^6 motile spermatozoa prior to freezing, while 30×10^6 motile spermatozoa were provided prior to freezing of semen used in Herd 2. We suspect that differences in fertility due to packaging method would not have been as great in Herd 1 if more spermatozoa were included per inseminate. Regardless of the reason(s) for the difference in fertility of semen from Bull C in the two herds, it is obvious that factors other than method of packaging *per se* can have marked effects on fertility.

Other techniques have been utilized for packaging and/or freezing of bovine semen. Some investigators have frozen semen in pellets by placing small drops of extended semen into small depressions on a block of dry ice, or in steel plates suspended in liquid nitrogen (LN) vapor. The comparative fertility of semen frozen in pellets, ampules, or straws in some studies is shown in Table 3. Again, the influence of packaging methods on fertility have been inconsistent.

Risks of Contamination

Pellets have not gained acceptance in the United States and Canada because of severe limitations in handling and distribution. Although pellets can be labeled,⁸⁰ permanent identification has presented some difficulties and the semen is still exposed to LN. Therefore, pieces of the pellets can be transferred to other pellets while in LN storage, creating a potential for mixing semen from different bulls.^{47,56} Stewart⁸⁹ conducted an extensive series of experiments related to storage of pellets and concluded:

1. Pellets can become contaminated with organisms present in LN.
2. Pellets can "pick up" spermatozoa in LN. The occurrence was rare and the spermatozoa "picked up" were nonmotile.
3. Organisms from contaminated pellets are shed into LN and can contaminate pellets in a different canister.
4. Spermatozoa became attached to forceps during handling of pellets.
5. Straws stored in the same LN with pellets became contaminated externally.

Thus, the potential for spread of disease is excel-

TABLE 3 Fertility of Spermatozoa Frozen in Pellets, Ampules, or Straws

Pellets		Ampules		Straws		References
% NR	Inseminations	% NR	Inseminations	% NR	Inseminations	
68.3	467	56.9	545	--	--	62
70.4	440	65.4	1,216	--	--	82
59.0	1,872	--	--	68.5	1,993	7
69.7	--	57.9	--	63.7	--	60
66.5	5,515	--	--	66.7	5,481	34
53.1	160	--	198	--	--	86
39.7	253	56.6	281	--	--	94
66.1	9,035	41.9	--	66.4	8,804	35
61.4	--	--	--	--	--	32
65.7	2,839	71.5	--	66.1	2,937	55
55.1	--	--	--	--	--	43
55.7	--	59.2	--	--	--	93
		68.5	--	--	--	

SOURCE: Adapted from Pickett *et al.*⁷⁰

lent and a pregnancy from the wrong bull possible.

A technique for freezing pellets in gelatin capsules was developed⁴⁷ to eliminate the possibility of contamination. However, the open end of the capsule was sealed with a drop of extender, which is exposed to LN and represents a potential source of contamination.

Straws can be sterilized externally⁶ but contamination of French straws and the powder used for sealing has been reported.⁴⁹ When properly sterilized and sealed, glass ampules are the most suitable package with respect to potential contamination of semen.

Contamination of ampules and straws can occur during thawing, primarily due to faulty seals. Improperly sealed ampules generally explode, particularly if they are stored under LN; while plugs generally pop from straws.⁹⁰ Ampules that have exploded may have been contaminated, but contamination is of little consequence, since the semen is unusable. When a plug pops from a straw, the semen should not be used, because the semen may have become contaminated. It is suspected that LN enters the straws during freezing or storage, and the seals are expelled due to vaporization during thawing. If nitrogen can enter the straw, other contaminants may also enter. Since expulsion of "plugs" is an occasional phenomenon, it is not known whether nitrogen entry is occasional or occurs routinely.

Influence of Package Size

The volume per inseminate does not appear to be an important factor in fertility of cattle, at least in the range currently utilized for AI. For example, American Breeders Service¹² reported 60- to 90-day nonreturns of 77.3 vs. 76.2 percent with 0.5- and 1.0-ml ampules, respectively, when the same total number of spermatozoa was included per inseminate. Hafs *et al.*³⁷ also reported similar 60- to 90-day nonreturn rates of 73.2 vs. 70.5 percent for insemination volumes of 0.9 vs. 0.5 ml, when all inseminates were frozen in 1.0-ml glass ampules and contained the same total number of spermatozoa.

Pickett *et al.*⁷⁰ reviewed results of fertility trials involving straws containing different inseminate volumes. Fertility was generally greater when semen was frozen in 0.5-ml as compared to 1.0-ml straws. However, many of the studies were confounded by the use of a different number of spermatozoa within each volume. Differences in fertility for 0.25- and 0.5-ml straws ranged from 0.6 percentage

points lower to 1.2 percentage points greater with the smaller straw. It would appear that where differences in fertility have been associated with inseminate volume, these have probably been due to concomitant changes in freezing rate, thawing rate, or some other aspect of seminal processing and/or handling.

SEMINAL PROCESSING PROCEDURES

Incubation of Raw Semen

A limited number of studies have been conducted to evaluate the influence of incubation of raw semen (herein designated as holding) prior to initial dilution and cooling on the survival and fertility of bovine spermatozoa. Rajamannan *et al.*¹⁷ reported a gradual increase in post-thaw motility as holding time at 26°C was increased for up to 60 min. Furthermore, the fertility of semen from two low fertility bulls was increased when semen was held for 20 min at 26°C compared to that of semen not held. In contrast, Jondet⁴⁰ obtained similar fertility (69.6 vs. 68.5 percent nonreturn) when semen was diluted and cooled immediately vs. held for 15 min at 32°C. In a preliminary study at our laboratory, a total of 14 of 26 heifers (54 percent) conceived when bred with unincubated semen compared to 12 of 26 (46 percent) inseminated with semen held for 20 min at 37°C. In this study, each inseminate contained only 10×10^6 motile spermatozoa prior to freezing. Elliott *et al.*²⁵ and Ennen *et al.*²⁸ both found no advantage to holding raw semen on post-thaw motility. In fact, post-thaw motility tended to decrease as holding time increased. Until additional investigations are completed, holding of raw semen from fertile bulls, prior to the initiation of cooling, is not recommended.

Initial Dilution

Bovine spermatozoa may be damaged by cold shock if they are cooled rapidly at temperatures above freezing. The consequences of cold shock include coiling of the tails with an accompanying irreversible loss of motility, an increase in the number of spermatozoa that are permeable to vital stains, a reduction in respiratory activity and glycolysis, loss of lipid, an influx of enzymes into the seminal plasma, an uptake of sodium and calcium, and a loss of potassium and magnesium by the cells.^{16,19,21,53,71,74,75,84,97}

Cold shock can be prevented by slow cooling in the presence of protective agents. Most extenders for bovine semen contain milk or egg yolk. The former contains casein, while the latter contains lecithin, lipoproteins, and phospholipids, which provide protection against cold shock.^{15,21,33,41,44,51,65} The concentration of protective substances present during the cooling of semen will depend upon the dilution ratio of semen to extender prior to cooling (initial dilution) and the concentration of these substances in the extender. Benson *et al.*¹³ obtained decreased post-thaw motility as initial seminal dilution was increased for semen subsequently frozen in ampules. Post-thaw motility averaged 36, 37, 34, and 32 percent when semen contained 1,000, 250, 40, or 20 x 10⁶ spermatozoa/ml during cooling to 5°C. The extender used for seminal dilution in this study contained a fixed level of egg yolk. Thus, higher rates of dilution were also associated with higher egg yolk concentrations during cooling. Zarazúa *et al.*¹⁰¹ studied the influence of initial dilution ratio on the post-thaw motility of spermatozoa frozen in plastic straws. In this study, the level of egg yolk in the extenders was adjusted so that 4, 8, 12, and 16 percent egg yolk were present within each dilution ratio. The results are given in Table 4. In this study, motility increased as the dilution ratio increased, regardless of the level of egg

TABLE 4 Influence of Initial Dilution Ratio and Egg Yolk Level during Cooling from 37 to 5°C on Motility of Bovine Spermatozoa Subsequently Frozen in 0.25-ML Continental Straws

Dilution Ratio	Egg Yolk (%) ^a				Mean
	4	8	12	16	
1:0.5	19.7	17.6	17.2	17.2	18.0
1:1	21.1	20.7	19.2	19.0	20.0
1:2	20.7	21.5	19.9	20.9	20.7
1:4	22.0	20.9	20.6	20.9	21.1
Mean	20.9	20.2	19.2	19.5	--

SOURCE: Adapted from Zarazúa *et al.*¹⁰¹

^aAll samples were adjusted to a final concentration of 16 percent egg yolk and 7 percent glycerol for freezing.

yolk. It was recommended that semen should be diluted with at least an equal volume of extender prior to cooling.

Cooling Time

The rate at which semen is cooled to about 5°C can have a significant effect on fertility. Erickson²⁹ compared the fertility of split samples cooled from 32.2°C to 3.3°C in 110 min vs. 4 h and used without freezing. The 30- to 40-day nonreturn rates were 74.5 and 80.6 percent respectively. Slower cooling has also been associated with greater post-thaw motility for spermatozoa frozen either in ampules or straws.^{13,28,52,54} In contrast, Weidler and Zaugg⁹⁸ found no difference in fertility for semen cooled from 35°C to 5°C in 1.5 or 3.0 h, and Jondet⁴⁰ found no difference in fertility of semen cooled from 30°C to 5°C in 0.5, 1, or 2 h. In view of the results of laboratory studies, we suspected that bull fertility and/or the number of spermatozoa per inseminate were sufficiently high to mask any effect of rate of cooling on fertility in these studies. Although there are some contradictory data, we must recommend cooling times of 2 h or more. The National Association of Animal Breeders⁶³ conducted a survey among semen processors and found that 15 percent cooled semen in less than 1 h, 52 percent in 1.0 to 1.5 h, 10 percent in 1.5 to 2.0 h, and 23 percent in more than 2 h. From these results, we suspect that the desire to complete seminal processing and freezing on the same day that semen is collected has resulted in a cooling rate of convenience rather than on based upon sound experimental evidence.

Glycerolization

Once extended semen has been cooled to 5°C, it is customary to further dilute the semen so that the spermatozoal concentration is twice as great as that to be present in the final product. Final dilution is then accomplished by adding a volume of glycerol-containing extender equal to that of the extended semen. Glycerol is added to semen as a cryoprotective agent. The optimal concentration of glycerol depends upon several factors, including the type of extender and freezing rate. Generally, levels of approximately 7 percent glycerol are optimal for citrate-yolk and tris-yolk extenders, and levels of 11 to 13 percent for fresh and reconstituted skim-milk extenders.⁶⁹ Although

there are some contradictory data, many investigators have reported damage to spermatozoa as the temperature at which glycerolization occurred was increased.^{13,17,22,23,24,58,73} Thus, extended semen is generally cooled to about 5°C before glycerolization. A variety of procedures for the addition of glycerol-containing extender to the extended semen have also been evaluated. These include glycerolization in a single addition, stepwise addition at intervals of several minutes, or by allowing the glycerol extender to drip slowly into the extended semen.^{22,58,64,99} The influence of glycerolization method on post-thaw motility in these studies was inconsistent. Following a review of the available data, we⁶⁹ concluded that under certain conditions the stepwise addition of glycerol to bull semen may enable greater post-thaw motility. However, when present, this advantage has generally been small, and may not be reflected in increased fertility.^{31,39}

The glycerolization procedure is used to provide both the proper level of glycerol for cryoprotection of the spermatozoa and the desired concentration of spermatozoa in the extended semen. Ejaculates with a low spermatozoal concentration have posed a distinct problem when semen was to be frozen in 0.25-ml straws, since the use of a 1:1 initial dilution and an additional 1:1 dilution at glycerolization can result in too few spermatozoa per inseminate. Therefore, Zarazúa *et al.*¹⁰¹ studied the effect of glycerolization by dilution at ratios of 1:1, 2:1, 3:1, or 4:1 (extended semen:glycerol-containing extender) with extenders containing 14, 21, 28, and 35 percent glycerol, respectively. These dilution ratios resulted in a final level of 7 percent glycerol for all samples. No difference in post-thaw motility was observed due to glycerolization procedure. Thus, it would appear that lower dilution ratios (i.e., volumes) may be used for glycerolization, provided the final product contains the proper level of glycerol for optimal cryoprotection.

Number of Spermatozoa per Insemination

The influence of the number of spermatozoa per inseminate has been discussed elsewhere in considerable detail.^{68,70} It appeared that the number of live spermatozoa per insemination for optimal fertility was similar for semen frozen in straws or ampules.⁶⁸ In only one of seven studies with semen in ampules was fertility significantly greater with the highest number of spermatozoa studied.⁷⁰ However, in

all but one of the seven studies, actual mean fertility was higher with the highest spermatozoal number. The same trend was generally evident in eight studies with semen frozen in plastic straws.⁷⁰ When all of the studies on number of spermatozoa per inseminate were considered, there appeared to be a trend towards the use of fewer and fewer spermatozoa. If this trend continues using nonsignificant data, it is certainly possible, if not inevitable, that overall fertility will be reduced.

The optimal number of spermatozoa per insemination depends, in part, upon the fertility level of the bull. Hafs *et al.*³⁷ reported no influence of number of spermatozoa per inseminate, over the range studied, on the fertility of highly fertile bulls, while fertility of bulls of lower fertility decreased when spermatozoal numbers were reduced. Although this trend was not significant, it was similar to that reported by Sullivan,⁹² as depicted in Figure 1. From these results, it appears that for optimum fertility more spermatozoa are required per insemination when bulls are of low fertility. Thus, the fertility level of each bull must be considered in establishing the number of spermatozoa per insemination. Unfortunately, the established optimum is only valid for that bull under those circumstances.

Equilibration Time

Numerous investigators^{18,26,27,36,50} have established that

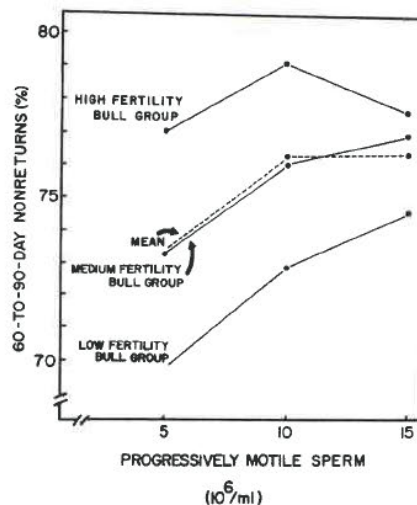


FIGURE 1 The effect of number of spermatozoa on nonreturn rate of Holstein bulls.⁹²

the fertility of spermatozoa frozen in ampules was enhanced when semen was stored with (equilibrated) or without (aged) glycerol for several hours after cooling prior to freezing. The optimal length of this period has varied, but generally is between 6 and 18 h.⁶⁹ Although it was previously thought that the presence of glycerol during equilibration was important, it was subsequently demonstrated that the period of storage or aging was the important factor, and that glycerol could be added at any time after cooling and before freezing, without influencing fertility.³¹

Laboratory investigation of optimal equilibration time is probably not fruitful, because it is suspected that the industry will adopt an aging or equilibration time of convenience rather than one based upon research results. Fortunately, it appears that extended equilibration or aging is not necessary for semen to be frozen in straws.

Freezing Rate

Some of the basic causes of cell death during freezing and thawing and methods for their prevention have been presented elsewhere.⁶⁹ While many of the principles of cryopreservation of living cells are well established, most procedures for the freezing of bovine spermatozoa have been developed empirically. Unfortunately, no fertility studies have been conducted to establish optimal rates of freezing for bovine spermatozoa. Rate of freezing can exert marked effects on sperm survival.⁷⁰ However, the optimal rate(s) of freezing is influenced by a variety of factors, including methods of seminal packaging, glycerol level, and subsequent thawing rates. From the available evidence it is obvious that spermatozoa are capable of withstanding a wide range of freezing rates. Nonetheless, procedures should be developed and utilized that minimize variation. This is important, as rate of freezing interacts with many other factors, and each of these factors must be controlled to obtain a uniformly high-quality product.

Storage

The key factor enabling the long-term preservation of bovine spermatozoa is low temperature. At one time, most frozen semen was stored in a mixture of dry ice and alcohol at -79°C . When stored at this temperature, the fertility of the semen ultimately decreased.^{59,83,88} However, Ström⁹¹

found no evidence of reduced fertility when approximately 60,000 inseminations were performed with semen in pellets after storage in liquid nitrogen (LN) at -195°C for approximately 1 to 1.5 yr. Cassou²⁰ reported no difference in fertility after 285,551 inseminations with semen in straws stored in LN up to 54 mo. If fertility of semen stored in LN is reduced with time, regardless of packaging technique some factor other than storage is responsible.

PROCEDURES FOR THE FIELD HANDLING OF FROZEN SEMEN

One of the major causes of low fertility associated with the use of AI is mishandling of semen by the inseminator. This fact has been confirmed by numerous studies conducted with full-time, professional inseminators closely supervised by AI organizations.^{66,67,72} Although data are lacking, mishandling of semen is probably more extensive today than at any time in the past, due to direct sale of semen to ranchers and dairymen who inseminate their own cattle, often with little training or supervision.

Principles for the field handling of frozen semen and specific recommendations for the field handling of bovine semen frozen in ampules or straws have been presented elsewhere in detail.^{14,67} Therefore, the following discussion will focus primarily on specific aspects of field handling that have, or are likely to have, profound influences upon fertility.

From the available evidence, it appears that seminal temperature should be maintained at -130°C or lower at all times.^{45,57,72,88,96} Although LN has a temperature of about -195°C , there are many opportunities for semen stored in LN to be exposed to damaging temperatures from the time it is shipped from the AI organization until it is thawed for use. One such opportunity is during transfer from the unit in which it was shipped into the field storage unit. Seminal temperature can increase rapidly during exposure to ambient conditions. The rate of warming depends, in part, upon the method of packaging. This is illustrated in Figure 2. Seminal temperature rose much more rapidly within straws than within glass ampules, because the straws contained a smaller volume of extended semen and because they had a much greater surface area-to-volume ratio. The greater increases in temperature were also associated with greater reductions in post-thaw motility (Figure 3). Straws placed in plastic goblets for field distribution are much less vulnerable to changes

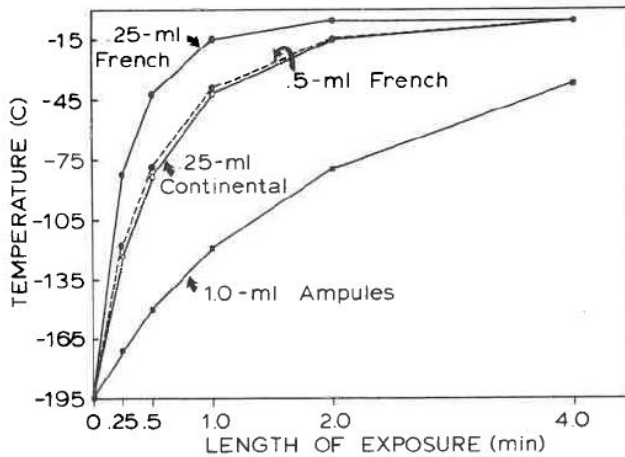


FIGURE 2 Temperature of semen within individual straws held by forceps or ampules clipped to metal racks (canes) during exposure to ambient ($20 \pm 0.6^\circ\text{C}$) conditions. (Each curve represents the mean of five replicates. The curve for ampules is a mean of the temperature of semen within the top and bottom ampule on the same rack.¹⁴

in temperature during exposure.¹⁴ Thus, we have recommended that AI organizations package straws in plastic goblets or other suitable protective devices for distribution and field storage. Rates of warming during exposure to ambient temperatures may also be influenced by factors such as wind velocity and the intensity of solar radiation. Thus, it is impossible to establish a safe interval for the transfer of semen from one unit to another. The most reasonable approach is to transfer the semen as quickly as possible, out of the wind and sunlight.

Semen may also be exposed to damaging temperatures each time it is raised to the neck of a field unit to permit removal of an individual straw for thawing. The temperature within the neck of a typical field unit is much higher towards the top of the tank (Figure 4). Since spermatozoa will be damaged at these temperatures, the canister containing the semen should be raised no higher than absolutely necessary, and the individual straw removed for thawing as quickly as possible.

One need not be nearly as concerned about the tempe-

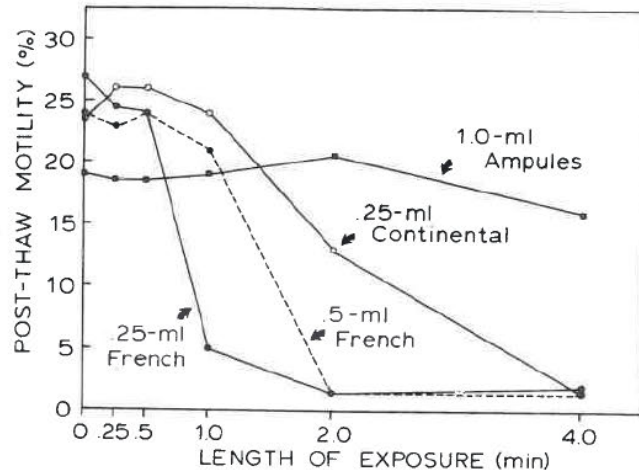


FIGURE 3 Influence of exposure to ambient ($20 \pm 0.6^\circ\text{C}$) conditions on the post-thaw motility of spermatozoa within individual straws or ampules on racks. (Semen in straws was thawed in water at 37°C , while ampules were thawed in iced water. Each value represents the mean of duplicate samples from each of vice bulls.¹⁴

rature of semen within the straw being removed as with the other semen in the same canister or goblet. This semen is exposed to elevated temperatures each time another straw is removed for thawing. The damage from exposure to elevated temperatures is additive. In addition, unless sufficient time is allowed between exposures to permit the semen to cool completely, higher temperatures will be reached during subsequent exposures.

It is obvious that the temperature reached by semen during exposure to the neck of a field storage unit depends upon the height to which it is raised, length of exposure, level of LN in the tank, and the intervals between exposures. Therefore, semen should be raised no higher than necessary to facilitate removal of a straw for thawing, and removal should be executed as quickly as possible. Since, under normal circumstances it is impossible to maintain a full LN level at all times, inseminators must be instructed to allow more time between exposures when the LN level is low.

Procedures for thawing semen can exert marked influ-

ences upon fertility. After reviewing the available evidence, Pickett⁶⁴ concluded that semen in glass ampules should be thawed in ice water. In contrast, the quality of semen frozen in plastic straws has consistently been enhanced in laboratory studies by rapid thawing in warm water.^{3,10,11,28,78,79,100} On the basis of fertility trials conducted at our laboratory, we currently recommend that semen in plastic straws be thawed in water at 35°C.¹⁴

Thawing in warm water is not difficult, but several precautions should be exercised. Spermatozoa are damaged by excessive heating. In one study, spermatozoa in 0.25-ml Continental straws were removed from LN and plunged into water at 35 to 60°C for 5 min, and evaluated immediately. Motility declined precipitously at temperatures above 45°C. Temperatures below 45°C may also be detrimental. For example, high temperatures can stimulate motility briefly, but are then followed by rapid cell death. Also, it has been shown that rabbit spermatozoa incubated for 2 h at 38 or 40°C were equally capable of fertilization, but fertilization by sperm incubated at 40°C was followed by high rates of embryonic mortality.⁹⁵ Thus, there may be considerable damage to spermatozoa warmed above body temperature.

Damage to spermatozoa thawed in water at above body temperature can be prevented by timing the thawing process very carefully. At the same time, it would be futile to recommend a thawing method that was so difficult to employ that it would greatly increase the opportunity for mishandling during and after thawing. Under such circumstances, fertility might be lower than with a method that was less than ideal but more easily accomplished. In view

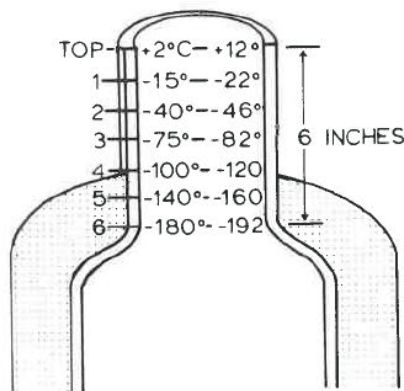


FIGURE 4 Typical temperature range in the neck of an LN field storage unit.⁸¹

of the extremely detrimental effects of excessive heating, and the fact that post-thaw motility has generally been only slightly enhanced at temperatures above 35 to 37°C, we currently recommend that semen in straws be thawed in water at 35°C.

Spermatozoa warmed to higher temperatures ($\sim 35^{\circ}\text{C}$) after thawing are susceptible to cold shock.^{9,85} Therefore, we¹⁴ recommended that thawing in warm water be carefully timed to permit thawing without causing further elevation in seminal temperature.

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