

The Integrity of Frozen Spermatozoa

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FUNDAMENTALS OF THE
PRESERVATION OF SPERMATOZOA

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From time eternal the mysteries and intricacies of sexual reproduction have been pondered. Enter man, the manipulator, into the normal process of reproduction of self or the beasts that serve him; enter artificial means of semen collection; enter collected semen; enter preservation in the liquid state; enter cryopreservation; enter artificial insemination; and enter challenges, opportunities, problems, and questions. Accomplishment in the field of physiology of reproduction has been made to an extent that would stagger the imagination of those not working in the area. But the immense perturbation that man has introduced into the process is as unprecedented as it is relentless and cannot now be checked save ignoring several centuries of hard-won understanding. We cannot reverse this trend of events, but we should have the power of discernment to use appropriately the fruits of our research in proper perspective. Goals in animal research have been directed to animal improvement for the betterment of mankind. Many of our findings are, however, directly applicable to the human, although we have no control at present over their use in this context either for good or ill. Research is turning intensively to the very basics, propelled headlong by applications beyond the limits of our current understanding. Our only recourse is to endeavor to recognize very early the scope of our impact, seek true answers, and apply knowledge cautiously with wisdom and vision for the future.

The purpose of this paper is to list and review several of the factors, steps, problems, and questions that may be important either to animal or man in the process of reproduction from sperm collection through fertilization. During this conference, drawing from the principal speakers and discussants, it is hoped that

definitive answers with appropriate data will shed insight to the many questions raised, and at the same time pose questions to guide future research important to reach our goals. Only selected references will be used to illustrate some diversity of opinion.

SEMEN COLLECTION

The artificial vagina is the preferred method of semen collection in most species. It provides for the proper temperature, pressure, and lubrication to simulate natural mating.⁷⁹ Care should be taken to maintain sanitary conditions. Electroejaculation of semen is used when animals are not trained for the artificial vagina or are physically unable to mount.⁴⁷ Larger volume, resulting in lower sperm concentrations with comparable or somewhat decreased fertility of bull semen, is obtained. Electroejaculated ram semen has less uniform ejaculates, lower quality, and lower fertility than semen collected with the artificial vagina.^{126,182}

Collecting semen too frequently causes a decrease in concentration and volume per ejaculate, but increases the total number of sperm collected per unit time. Variations do exist from species to species.⁷⁹ The maximum potential daily sperm output has been estimated as 12.8 billion (mature Holstein bulls), 5.3 billion (young shorthorn bulls), and 31.3 billion (2-yr-old Yorkshire boars).³⁴

Sexual preparation and stimulation by allowing a single false mount increases sperm output by 50 percent, while three false mounts increases production by 100 percent. Prolonged restraint before mounting has a similar effect. Changing teasers and environment also increases sexual interest and semen quality and quantity.¹⁸⁹

INITIAL SEMEN QUALITY

Volume of semen varies with age, species, and individual animal and is used along with sperm concentration and motility to determine final dilution rate for freezing. One study showed a significant ($r = -0.170$ and -0.111) correlation between volume and fertility.²⁴

Many researchers have investigated the relationship between initial sperm concentration and fertility.

Initial sperm-rich cell concentration of boar semen gave the highest correlation to fertility of all characteristics studied ($r = 0.61$).¹⁹⁶ Similar results have been reported for the stallion.²¹⁸ However, no significant correlation between sperm concentration and fertility in ram semen has been reported.^{97,233} Several investigators ^{24,25,172} reported significant correlations between initial sperm concentration and fertility ($r = 0.216$ to 0.353) for bull semen. However, others ^{39,40,50,92} reported no significant correlation between concentration and fertility. In general, initial concentration does not appear to be a practical method for predicting fertility, but definitely influences the chemical environment that may be important to the cell.

Viability is often assessed by estimating percentage progressively motile cells using a microscope or closed-circuit television. Other types of electronic and photographic equipment have been used. Abnormal motion patterns indicate cold shock or chemical shock.⁷⁹ Observations of sperm velocity, survival, and swimming patterns using photoelectric methods have been correlated to fertility.²²⁰ Percentage of initial motility has also been correlated to fertility ($r = -0.218$).²⁴ Significant correlations between post-thaw motility and fertility have also been reported.¹⁵⁵ Various vital stains and morphology tests have been widely used to assess semen quality. In general, when there are less than 20 percent abnormal sperm cells, fertility is not affected, but as the percentage of abnormal sperm cells increases, fertility decreases.³⁴ More recently, acrosome morphology of thawed, incubated semen has been shown to have a high correlation with fertility ($r = -0.81$) based on 90-day nonreturn rates.¹⁸⁰ Several metabolic tests have also been utilized, such as methylene blue reduction, oxygen uptake, pH change, fructose utilization, and lactic acid production. Investigators ²⁴ reported significant correlations with oxygen uptake ($r = -0.267$) and methylene blue reduction time ($r = -0.227$) with fertility, while others ¹⁷² reported no significant correlations with these tests.

Semen Chemistry

The constituents of semen are numerous and varied--choline, citric acid, fructose, inositol, ergothioneine,

various ions, and other substances not found in large quantity in the rest of the body.³⁴ The main constituents which have been studied, which vary greatly, include inorganic phosphorus, Na, K, Ca, Cl, total protein, reducing substances, and phosphatases.

Minerals

Inorganic phosphorus is used in most bovine semen extenders, but has been found to depress motility and oxygen consumption in higher concentrations.¹²¹ Early studies indicated that fertility was not correlated with levels of inorganic phosphorus in the bovine,⁵⁵ but later work⁵³ established that levels of inorganic phosphorus were correlated with combined levels of Na and K and were not significantly related to fertility.

Many mineral substances are found throughout the male tract and in semen, and come from both the seminal plasma and spermatozoa.¹²¹ Sodium, potassium, and calcium are the bulk cations, with many trace elements (boron, magnesium, iron, copper, etc.) existing in very small concentrations.¹⁸⁹ In an early review,¹²¹ concentrations of Na and Ca were believed to be higher in seminal plasma and K concentrations higher in the sperm cell, with the latter being confirmed by others.²³⁵

Potassium was found to depress motility in bull semen,^{22,199} and a higher concentration was found in epididymal semen than in ejaculated semen (K:Na ratio 1:1 in epididymal plasma, 1.0:2.5 in seminal plasma). This accounted for decreased motility in K-rich medium and epididymal sperm. The concentration appeared to shift in the ejaculation process with added seminal fluids, and the increased Na concentration initiated motility in ejaculated semen.^{121,199} High concentrations of electrolytes in semen extenders were found to have a damaging effect on sperm.^{21,96} In ram semen, the addition of K increased metabolism. Calcium depressed O_2 uptake and fructose oxidation. However, the addition of 0.5-5.0 mM KCl stimulated the metabolism of both recently ejaculated and thawed spermatozoa.¹⁴²

Some attempts⁷³ have been made to correlate minerals to fertility. The data suggests that in bull, boar, and turkey spermatozoa, a positive correlation exists with K and P and a negative correlation exists with Ca concentrations found in seminal plasma and fertility.

Total Protein

Many studies have involved the total protein content in semen. It is estimated that 90 percent of the total N present in seminal plasma is protein.¹⁸⁹ Seminal plasma protein was found to be approximately 14.4 percent N.¹⁰⁴ Various figures have been given for the actual protein content in bovine semen, and they all were very close: whole semen, 7.32 g percent; seminal plasma 7.04 g percent.²²³

It was shown that total protein content of bovine seminal plasma was not significantly correlated with fertility. It was, however, related to reducing substances, Ca and Cl concentrations,⁵³ and semen volume, but not to sperm cell concentration.¹⁰⁰ Those species (bull, ram, goat, and man) with higher seminal plasma protein concentrations responded to the freezing procedures more readily than those species with low seminal plasma protein concentrations (boar, stallion, and turkey).

Reducing Substances

The reducing power of sperm has long been used as an indicator of semen quality,¹²¹ and several reducing substances have been found in semen: fructose, ascorbic acid, sulfhydryl compounds, and sulfite.¹⁸⁹ Fructose is the principal reducing compound and is actively metabolized by sperm cells. This reducing substance is derived solely from the seminal vesicles,¹⁶ with various estimations made as to its concentration: 376-1,062 mg percent,¹²¹ 280-779 mg percent, and 26-872 mg percent.¹⁷⁷ A significant correlation between initial fructose level and fertility was reported.⁴⁹

Immotile sperm cannot use fructose, so a positive correlation between fructolysis rate and motile sperm concentration was expected and confirmed.^{18,51}

A formula for predicting fertility level using fructolysis was devised, and it was found that bulls with a high fructolysis index had a conception rate 5.9 percent higher than bulls with a low index.¹⁶⁴ However, negative correlations between reducing substances in seminal plasma and fertilizing capacity has also been reported.^{53,73} Sperm cell concentration and volume are inversely related to concentration of seminal fructose.⁵¹ Therefore, a

high fructose concentration may not indicate fertilizing ability.¹²¹

Enzymes

The presence of phosphatases was first demonstrated in human semen in 1935 and bovine semen in 1945.¹¹⁹ Acid and alkaline phosphatase activity is intermediate in the bull, while acid is high and alkaline low in the human.¹⁴ Both alkaline and acid phosphatases were positively correlated with sperm cell concentration, volume, and motility.^{84,173,179,200} Only alkaline phosphatase was correlated positively with fertilizing capacity,²⁰⁰ while both alkaline and acid phosphatase showed a positive correlation in another study.⁷³ Several enzymes have been added to semen extenders, and it has been suggested that their inclusion enhances fertility.

Certain cellular components are released when cells are damaged. Measurements of GOT (glutamic oxaloacetic transaminase) and LDH (lactic dehydrogenase) are two constituents that can be used as a possible indicator of fertility or damage.^{70,73,143}

BUFFERS OR EXTENDER MEDIA

An ideal extender for semen should:

1. maintain proper osmotic pressure and electrolytic balance to ensure minimal salt effects;
2. have constituents with metal-complexing properties that could be effectively utilized to bind heavy metals harmful to sperm cells;
3. have a pK between 6 and 8, preferably 7, and have buffering capacity so deleterious shifts in pH can be prevented as lactic acid is formed and cooling occurs;
4. contain constituents that provide protection against the harmful effects of cooling and freezing;
5. be stable, resisting enzymatic and nonenzymatic degradation;
6. contain antibiotics that inhibit bacterial growth;
7. substantially increase the volume of semen so that multiple inseminations can be performed; and
8. provide an environment in which the metabolic activities of the sperm can continue.

Ions and Zwitterionic Buffers

The increase in intracellular salt concentration due to the removal of water from the cell during ice formation is a major cause of sperm mortality during freezing. The protective action of cryoprotectants such as glycerol is largely attributed to their salt "buffering" capacity, thus electrolytic damage as the water freezes out is minimized. It has also been reported that extracellular salts have a deleterious effect on spermatozoa.¹²¹ Potassium and sodium have been shown to decrease the motility of bull spermatozoa.^{37,236} Excess or insufficient calcium ions also adversely affect motility.²³⁷ The heavy metals, such as Cu, Zn, Ag, Pt, Ni, Al, and Au (in decreasing order of potency), also decrease the motility of human spermatozoa.⁹⁹

The zwitterionic buffers MES,¹¹⁸ PIPES, TES, HEPES,⁷⁸ BES,⁸⁹ MOPS, and TRICINE⁷⁷ have been used extensively as buffers for spermatozoa.^{6,7,8,9,12,25,41,42,57,60,70,236} The effect of binding heavy metals by BES and TES may explain their efficacy in decreasing the release of GOT, as is possibly the beneficial effect of sodium citrate and phosphate.⁶⁹

Since the development of the phosphate,¹⁴⁷ sodium citrate,¹⁸⁷ carbon dioxide bicarbonate,²¹³ and trisbuffers,^{22,41,42,57,60,236} little development of other buffers for preservation of bull spermatozoa has been reported. Many attempts have been made to improve these basic buffers by the inclusion of various additives such as gelatin, peptone, glycerol, glycine, glucose, and many other materials. Undefined materials, such as honey, coconut milk, tomato juice, alfalfa, and guarana leaves, have also been used with varying degrees of success.

The discovery that egg yolk in semen extenders has a beneficial effect on fertility^{147,148} led to its widespread use in bull semen extenders. Early researchers reported that egg yolk aided the sperm cell in resisting cold shock.^{21,106,107} However, the discovery¹⁶³ that bull semen could be successfully frozen in extenders containing glycerol diverted researchers from studying the protective properties of egg yolk. Further research indicated that glycerol was not essential for freezing spermatozoa under certain conditions^{15,65} and actually decreased fertility when added to buffers for chickens, turkeys, and boars.^{26,36,71,158,160}

Subsequent studies have shown that the lipoprotein fraction of egg yolk may be responsible for the protection

of spermatozoa against cold shock. This is accomplished by minimizing the loss of lipids from the sperm during freezing.³⁴

While amino acids do not provide significant protection to spermatozoa,¹³ peptides in certain concentrations were found to prevent damage to cells.

Antibiotics

Semen extenders are a good culture medium for bacteria, which may reduce fertility or adversely affect the inseminated female. Microbial contaminants have been linked to low fertility and conception rates, abortion, reproductive failure, endometritis, salpingitis, and other reproductive disorders.⁸³ Until recently, penicillin, sulfanilamide, streptomycin, and neomycin were the main antibiotics used to control bacteria that contaminate semen ejaculates.^{5,6,46,48,238} Recently these have been replaced by Linco-Spectin, due to its superiority in controlling mycoplasma, and Vibrio.⁸³ The increased efficiency in controlling microbial contaminants by using Linco-Spectin is obtained without loss of fertility.⁹

pH - pK

Changes in pH may occur in addition to an increase in salt concentration during freezing, due to precipitation of either ice or salt. To date, there is virtually no quantitative information available on the measurement or change in pH at or below the freezing point for a mixed solvent system (an extender for biological material) incorporating a cryoprotective nonelectrolyte such as glycerol or DMSO.

Measurement of pH at low temperatures is difficult. As early as 1932,⁵⁴ Finn, using a glass electrode, showed that as the temperature of muscle tissue was lowered from +15° to -4°C, its pH increased on the order of 0.02 units per degree Celsius. He also stated that when freezing commenced, concentration of the aqueous phase took place, producing a further change in pH. He showed that the pH of this liquid portion of frozen muscle tissue decreased with temperature below the freezing point.²¹⁰

A solution of concentrated milk, collected by

centrifuging an ultrafiltrate, showed a pH shift from 6.7 to 5.9 during freezing.²¹⁰

The pH of freezing aqueous salt solutions^{214,217} showed that sodium phosphate solutions with a pH of 7.0 at 25°C decrease in pH during cooling (approaching a pH of approximately 3.5 near the eutectic point, -9.9°C), while potassium phosphate solutions increase in pH during cooling (approaching a pH of approximately 7.5 near the eutectic point, -16.7°C). The differences in pH are due to low solubility.

Van den Berg²¹⁵ described a modified electrode for direct measurement of pH in frozen materials at temperatures as low as -30°C. Electrodes that can be used to measure pH at temperatures as low as -20°C are commercially manufactured today.

The pH of bull semen was estimated to be between pH 7.0 and 7.5.²²⁶ Later studies⁸⁵ stated the pH range for semen of fertile bulls may be quite wide and lie between 6.4 and 7.8 with normal semen slightly acidic and never more alkaline than pH 7.3.¹²⁷ Present-day bovine semen extenders vary in their pH specification, ranging between 6.5 and 7.2.^{58,62,69}

It has been speculated that quick freezing and thawing may not allow the biological material to be exposed to critical pH and salt concentration long enough for inactivation to occur.²⁴ It is also possible that at these low temperatures biological material may be less sensitive to pH changes than at body temperatures.

DILUTION

Time and temperature of dilution vary with species and are influenced by whether the sperm are easily cold-shocked, life expectancy of the cells, and biochemical changes of the cells in seminal plasma. Turkey semen does not cold-shock and has a life expectancy of only a few hours *in vitro*, so it can be diluted at 5 to 37°C immediately after collection. Semen of many species, such as bull, ram, stallion, and goat, is routinely extended immediately after collection at 37°C and cooled slowly to prevent cold shock. Bull semen held in its own seminal plasma for 20 min at 26°C before dilution resulted in higher fertility than semen extended 0 min at 26°C.¹⁷¹ Several reports^{71,72} show that slow cooling (1-2 h) to 22°C is important to maintain fertility in the use of frozen boar semen. A protein layer, important to

fertility, is developed on the sperm head from the seminal plasma during this period of time.¹⁴⁶

The dilution ratio for freezing varies with species, freezing container and technique, thawing and insemination techniques, and volume and sperm concentration needed for fertility. High and low concentrations of sperm cells may be frozen successfully, depending on technique and species.

It has been shown⁵⁸ that the extender with the lowest pH consistently gave nonreturn rates equal to or higher than the rates for the control. Studies demonstrated²⁰¹ that the least damage before freezing occurred in 0.20 M medium (containing tris) of pH 6.5, and the highest percentage of post-thaw motile spermatozoa was observed in an extender adjusted to pH 6.50 and containing 0.20 M tris, 6.4 percent glycerol, and 1 percent fructose.

The effect of pH upon motility, velocity, and metabolism of bull sperm has been demonstrated by numerous works.^{19,37,103,136,188,190,208,219,234} The observed responses varied markedly with temperature, media composition, and from sample to sample.

In selection of proper pH of an extender, one should be aware of the changes that occur during cooling and freezing. As concentration increases and the eutectic point is reached, the sample is approaching the pK value reflecting large changes in pH.

Although the osmolarity of semen and seminal plasma is similar to that of other biological fluids, sperm can tolerate considerable deviation in osmotic pressure. Motility may actually be enhanced in hypotonic solutions; however, fertility is maximal when the extender is isotonic.³⁴

Concentration and Dilution Effects on Post-Thaw Survival and Fertility

Ram semen frozen at very high concentrations (1.5×10^9 cells/ml) did not survive as well as semen frozen at lower concentrations.^{109,110} Likewise, semen frozen at an extension of 1:4 has better fertility than semen frozen 1:2.^{209,221} Highest fertility was reported for cell suspensions frozen at a concentration of 666×10^6 cells/ml.¹⁰⁸

A concentration of 1×10^9 cells/ml had a reported fertility of 76 percent¹³⁴ and post-thaw motility for

samples frozen at $3-5 \times 10^8$ cells/ml was enhanced if seminal plasma was removed.³⁵

Frozen chicken semen at concentrations of 0.750×10^6 cells/ml resulted in high fertility.²²⁵ Shaffner¹⁹² reported 100 percent fertility for one treatment of semen frozen at 10^9 cells/ml. Post-thaw motility was depressed when cells were frozen at 0.330×10^6 cells/ml as compared to a concentration of 600×10^9 cells/ml.²²⁴

A concentration of 2×10^9 turkey sperm cells/ml has been frozen with a fertility of 50 percent.²³⁹ Reports of previous work showed a uniformly low fertility for concentrations between 3.5×10^9 cells/ml²⁶ and 1×10^9 cells/ml.¹¹⁷

Bull semen has been frozen successfully at high or low concentrations. Three studies have reported successfully freezing bull semen at 500×10^6 cells/ml.^{157,211} As extenders and methods have improved, reports of a dilution effect have diminished.

Boar semen showed depressed motility when frozen concentrated ($500-1,000 \times 10^6$ cells/ml)⁶⁸ or dilute ($15-30 \times 10^6$ cells/ml).¹⁸¹ However, in one study,⁶ spermatozoa frozen in high concentration (600×10^6 cells/ml) resulted in 85 percent fertility.¹⁸⁶

COOLING

Rapid decreases in semen temperature from 37° to 5°C cause cold shock, a damage to the sperm cell caused by changes in gas solubility, metabolism, fluid viscosity, and chemistry. However, prolonged exposure of semen to higher temperatures maintains greater metabolic activity and earlier death of spermatozoa.¹⁸⁹ Semen from most mammalian species is subject to cold shock. The optimum cooling rate is one that prevents cold shock but is fast enough to maintain healthy viable sperm cells.

Lipoproteins and lecithins, such as found in egg yolk and milk, provide protection from cold shock when added to the semen before cooling.¹⁸⁹ Extender composition and pH have both been reported to influence the cold-shock susceptibility of boar spermatozoa as reflected by motility.^{20,141,167,168} Changes occurred in acrosome structure in spermatozoa as a result of cold shock. A proper cooling rate to freezing temperature is important to preserve the viability and fertility of bovine semen.¹⁸⁹ However, human sperm are not susceptible to cold shock.¹⁹³

CRYOPROTECTANTS

Since the discovery of glycerol as a cryoprotective agent for mammalian spermatozoa, much effort has been devoted to determining its protective mechanism. Several well-documented investigations have been conducted to determine its protective mechanism. These include: (a) decrease in high concentration of solutes, (b) maintenance of pH, and (c) change in liquid-ice phase. Several studies using highly sophisticated methods of evaluation and taking into consideration (a) molar solubility excess, (b) the volatility factor, (c) protection coefficient, (d) free energy of hydration, (e) hydrogen donors, (f) hydrogen binding, and (g) interference between donor and acceptor groups have been reported in an attempt to relate protective qualities, but with little success. Many small-molecular-weight penetrating compounds, referred to as colligative agents mimicking glycerol, have been studied along with other classes of materials, which include sulfoxides, N-oxides, and acetamides.

Some investigators reported that some polymeric solutes afforded protection to red blood cells. These include polyvinylpyrrolidone, polyethylene glycol, gelatin, starch, polyvinyl alcohol, poly-N-methyl glycine, and polyvinyl methyl sulfoxide.

The empirical application of colligative and/or polymeric protectors on freezing of cells becomes an endless endeavor. The effect of empirical application is difficult to interpret, because each compound seems to exert different effects in different concentrations, in different biological fluids, on different cell types, and by each on freeze rate. Also, there exist interactions between colligative and polymeric compounds that are difficult to separate. Polymeric compounds in this text refer to larger-molecular-weight proteins, lipids, or combinations. To this author's knowledge, there have been no reports of successful freezing of spermatozoa with any colligative compound in the absence of these materials. However, successful freezing has been demonstrated in the absence of colligative compounds, but is generally enhanced by their addition.⁷² A total of 63 colligative and more than 100 polymeric compounds (either pure or dialyzed or nondialyzed from body fluids with or without further fractionation) have been tested in an appropriate buffer solution. Data showed that no colligative compound was effective in the absence of polymeric material, but several, including

glycerol, DMSO, 1,2-propanediol, 1,3-propanediol, ethylene glycol, triethylene glycol, 1,4-butanediol, and N-methyl acetamide, enhanced the recovery of spermatozoa in the presence of appropriate polymeric material. Egg yolk or its derivatives was the only polymeric material placed in an appropriate buffer solution that afforded appreciable protection in the absence of colligative agents. Other protein or lipoprotein materials, such as those in blood, milk, or other body fluids, were effective, but only in the presence of colligative materials.

Regardless, some success in the freezing of spermatozoa can be obtained by placing the cells in practically any type of isotonic solution containing lipoproteinaceous material with appropriate concentrations of several different colligative compounds. The time, temperature, rate of addition, and concentration of these compounds have been studied with many conflicting results. Addition of glycerol to bull semen at 5°C has been reported to be better than at 10 or 20°C,³⁰ while others⁷⁵ reported no difference between 5, 10, 20, and 37°C addition of glycerol. The same conflict of opinion is reported for ram spermatozoa.^{33,108} Glycerol addition rates of 1 to 16 parts over periods of zero to several hours, as well as final concentrations of 2 to 20 percent (v/v), have been studied with conflicting results. In general, differences due to time, temperature, rate, and concentration of glycerol addition vary with species, extender, freezing, and thawing techniques. A general recommendation is to add glycerol to semen slowly with extender at 5°C: 7.0-7.5 percent in yolk citrate, 10-13 percent in heated skim milk, or 7-10 percent in heated homogenized milk.¹⁸⁹

Semen equilibration time after addition of glycerol has been studied with conflicting results. Higher fertility has been reported¹²² with bull semen equilibrated for 18 h rather than 2 h and the exact reverse reported by others,^{124,138} while still others⁷⁵ reported no difference among 30 min, 4 h, and 18 h equilibration. In general, optimum equilibration time has not been established, but varies with species, extender, and techniques employed and is presumed to be shorter rather than longer.

PACKAGING OF FROZEN SEMEN

The three most common packages for frozen spermatozoa

are the ampule, the straw, and the pellet. Most of the relevant fertility data compares bovine spermatozoa frozen in ampules or in straws. The results from these studies indicate that fertility is virtually the same for these two packages. Good recovery can result from any of these three packages, and the choice between them is largely influenced by personal preference. Listed below are the advantages and disadvantages of the three common packages.

Ampule

Glass ampules are commonly available, they lend themselves to convenient flame sealing, and they are easily labeled. The thickness of the glass makes very high cooling or warming rates difficult, but these rates are seldom necessary. Because of their shape, ampules are not efficient in terms of storage space.

Straw

Plastic straws are not commonly used in general laboratories, but their use is widespread in the cattle-breeding industry. They can be readily labeled for identification and sealed to exclude contaminants. Straws lend themselves to high cooling and warming rates, if such rates are necessary. They also offer more efficient use of storage space than ampules, an important factor for long-term storage. The straws waste little extended semen because an insemination can be made directly from them, avoiding significant losses from transferring to an intermediate pipette before insemination.

Pellet

Pellets are formed by placing a drop of extended semen directly onto the surface of dry ice or, in rare instances where very high cooling rates are desired, on liquid nitrogen. The frozen spermatozoa are placed in suitable containers after freezing, so positive identification of each unit is more difficult than with ampules or straws. Special precautions are necessary to avoid undesirable contamination during storage. The cooling rate is very consistent, because the surface of

dry ice is always the same temperature. On the other hand, it is difficult to vary the cooling rate of pellets, although some variations of cooling rate can be achieved by varying the pellet volume.

STORAGE

Use of dry ice or liquid nitrogen storage temperatures has extended the life of frozen semen; these have been compared repeatedly. Early work indicated only a slight difference in nonreturn rate, favoring liquid nitrogen.¹⁵² In later trials, nitrogen yielded higher nonreturn rates than dry ice and is believed to reduce possibilities of errors in handling.^{155,212}

While semen stored in liquid nitrogen is more resistant to exposure than that in dry ice,^{64,155} repeated exposure to warm temperatures and solar radiation is suspected of decreasing fertility of the semen.¹⁴⁹

When dry ice was the common method of storing semen, the conception rate from ampules warmed to -55°C and recooled to -79°C showed a marked drop from 70 percent to 28 percent.¹¹³ Others found severe damage if semen was allowed to partially thaw, then refreeze.¹⁵⁵ Rising storage temperatures, rather than original storage temperatures, are responsible for motility loss in human semen as well.¹⁴ Researchers agree that exposure of any frozen semen should be kept to a minimum, whether stored in liquid nitrogen or dry ice.

Fertility

Early low-temperature storage allowed for only short-term use of bull semen. Once dry ice was used, semen was stored from 3 mo to 4 yr without a large decrease in conception rate.^{52,113,114,130-132} Liquid nitrogen is a more recent advancement, and results have been extremely acceptable. Nitrogen was slightly favored over dry ice in early trials^{95,152,156,202} and produced conception rates nearly as high as that from fresh semen.¹⁵⁴

It has been suspected that some aging of the spermatozoa may occur if semen is stored for a long period of time and that this may be associated with embryo mortality and delayed returns.¹⁸⁴⁻¹⁸⁶ In some studies, semen stored at -196°C was consistently high.^{31,56,111,129,203}

Ram semen has been used with success after 5 yr storage,¹⁰³ and human semen has been preserved for up to 10 yr. Freezing and thawing procedures account for most of the loss of motility of human spermatozoa, but storage has also been considered to be the cause of significant declines in motility.¹⁶⁷

Genetic Degradation

Now that human semen is being frozen and stored, the problem of possible genetic degradation becomes more pronounced. It is agreed that cryopreservation is genetically innocuous¹⁹⁵ and that the possibility of genetic damage is extremely remote.¹⁹⁴ When frozen and stored semen has been used, fewer abnormal babies and spontaneous abortions have occurred than in the general population. In commercial cattle breeding, no evidence of abnormal offspring or skewed sex ratios have been attributed to the use of semen stored at subzero temperatures.

RATE OF FREEZING AND THAWING

Smith and Polge^{159,161,197} originally suggested that optimal recovery of bull sperm frozen in 1 cm³ ampules requires an average cooling rate of 1 to 2°/min from +5 to -15°C and 4 to 5°/min from -15 to -79°C. While this became the "standard" rate for freezing semen, later experimenters found other rates worked well also.^{12,17,27,32,43,63,88,91,98,105,112,139,161,178,222} Freezing in a liquid nitrogen freezing machine proved to be equal or superior to freezing at the "standard" rate with dry ice in alcohol.^{32,178} Plunging samples from +5°C into a -20°C bath and subsequently cooling at 25°/min gave equal recovery to the standard rate,¹⁷ as did immersion into baths held at -10, -20, and -30°C, but not -40°C⁸⁸ or directly into a -40°C bath,⁹⁸ or -45°C bath.¹⁰⁵ Equally good recovery resulted from controlled liquid nitrogen freezing from 2.5 to 3.0°/min from +5 to 15°C and 5 to 6°/min from -15 to 50°C,⁶³ or 1 to 5°/min from zero to -10°C and 4 to 20°/min from -10 to -30°C.²⁷ Cooling above -15°C at rates of 15 to 50°/min had little detrimental effect if rate was reduced to less than 30°/min between -15 and -30°C,⁹¹ although freezing faster than the standard rate caused rapid deterioration of semen after 24 h storage at 79°C.²⁰⁷

A critical temperature range, within which the greatest amount of damage occurs during "standard" cooling, is reported as -10 to -35°C .¹³⁹ The effect of damage within this range was reported to be minimized by equilibrating semen in diluent at 5°C for up to 18 h.^{123,161} This critical range is also characterized by sensitivity of bull spermatozoa to high cooling velocities above -27 or -30°C , but very resistant to high velocities below -30°C .^{42,161} Plunging into liquid nitrogen from -20°C results in practically no survival, but plunging from -27°C allows maximal survival.¹¹²

Thawing in ice water or water at 40°C or above is superior to thawing in 15 to 20°C water.^{140,153} using motility¹⁵³ or motility and acrosome retention,¹⁴⁰ while 60 to 90 day nonreturn rates show 5°C equal to 20°C and 40°C thawing bath,⁸¹ or to 5°C thaw when the ice cap is removed 45 s after immersion.²³

Freezing in smaller shapes, i.e., pellets and straws, has allowed much faster freezing and thawing rates due to a larger surface to volume ratio than that of ampules. The "standard" method for pellets is to drop extended semen on dry ice,¹³⁵ while for straws it is to suspend them singly on a rack in liquid nitrogen vapor.⁹⁴ Pellets and straws freeze much faster than ampules, and pellets never show supercooling. Pellets and straws frozen by standard methods give similar nonreturn rates.^{66,128} Varying the freezing rate of pellets by using volumes of 0.019 to 0.095 ml has very little effect on motility.²²⁷ Straws and ampules frozen by standard methods show similar motility when thawed in ice water, even though the 0.3 ml straw freezes at $108^{\circ}/\text{min}$ from $+5$ to -15 to -60°C .⁸ Semen frozen in 0.25 ml straws shows significantly better fertility than the same semen frozen in ampules.¹³³ Optimal freeze rate may depend on thaw rate used, since, with a 5°C thaw bath, straws frozen at $32^{\circ}/\text{min}$ from $+5$ to -15° to -60°C showed significantly poorer recovery than straws frozen $43^{\circ}/\text{min}$ from $+5$ to -15°C and $25^{\circ}/\text{min}$ from -15 to -60°C , while thawing at 75°C eliminated freeze-rate differences and produced significantly better recovery.⁶ Also, for straws frozen at $6^{\circ}/\text{min}$ ($+5$ to -15°C) and $12^{\circ}/\text{min}$ (-15 to -60°C) or $95^{\circ}/\text{min}$ ($+5$ to -15°C) and $53^{\circ}/\text{min}$ (-15 to -60°C), no freezing rate differences were seen.⁷ For semen extended in heated skim-milk-glycerol extender and frozen in straws, an increased percent of normal acrosomes^{230,232} and of progressive sperm motility^{231,232} was found as thawing bath temperature was increased;

this was also true for egg-yolk citrate-glycerol extender.^{175,176} Semen in straws survived better if frozen in dry ice-alcohol than in liquid nitrogen vapor when cooling rate is the same,¹¹⁶ perhaps due to more efficient heat transfer. Thus, as a general rule, fast freezing of straws is superior to slow freezing^{8,151,176} or shows no difference,^{7,8} while fast thawing is superior to slow thawing.^{1,2,7,8,151,174,175,230,232} The superiority of fast freezing is independent of thaw rate¹⁷⁶ or is significantly better only if a slower than optimal thaw rate is used.⁸

If rate within an applicable range is important (\pm few hundred degrees C per min), the only controllable method is one involving fast and efficient heat transfer. This can only be achieved by bath or thermal conductivity other than gas or vapor.

Freezing does not take place at a constant rate. The rate of cooling in a semen sample as it freezes is constantly changing due to two factors. The first factor is the change in phase from liquid to solid, occurring both abruptly at the freezing point and gradually until the eutectic point. The removal of the heat of fusion that allows this change in phase is aided by the greater thermal conductivity and smaller specific heat of ice than of water. The second factor, which can be controlled, is the way the sample is cooled. This includes the shape (geometry) of the sample, especially the surface/volume ratio; the contact to the cold surroundings; and control of temperature of the cold surroundings. Perhaps the disagreement in the literature as to the "best" method to freeze and thaw is due to repeatability problems, unreported differences in how the sample was cooled, or the effect of glycerol in widening the range of freezing rates giving acceptable results. Average rate of cooling over a wide temperature range is not an adequate description of how the sample was cooled.

Two basic ways of freezing by cooling in a bath are static freeze (bath fixed at a temperature colder than the sample) and dynamic freeze (bath cools, sample temperature follows bath temperature). In a dynamic freeze, the sample follows the bath until it crystallizes. During the freezing plateau the bath continues to cool. Once the heat of fusion has been removed, the rate of cooling is quite high until the sample temperature catches up with the bath. It is possible that this faster "catch-up" rate could injure the cells, especially if it occurs within the "critical temperature range" above -27°C .

There is no problem of the bath "getting ahead" of the sample when cooling in a static bath; however, the resulting cooling curve is nonlinear, while the cooling curve of a dynamic bath may be controlled.

One common "bath" is cold vapor of liquid nitrogen. It is used for static freezing by immersing samples in the static vapor above liquid nitrogen or for dynamic freezing by vaporizing liquid nitrogen at a controlled rate into a closed chamber in which nitrogen gas is circulated. Freezing curves produced by static vapor methods generally vary greatly from sample to sample within a freeze and especially from freeze to freeze. Since nitrogen gas has a low heat capacity per unit volume (1/1,500 of alcohol), a small amount of heat will warm the gas a great deal; the 80 calories of heat from freezing 1 cm³ of water would warm 1 liter of gas approximately 260°C. Thus, static vapor equilibrium is easily disturbed. In addition, the poor thermal conductivity of nitrogen gas (1/9 that of alcohol) impedes fast, efficient heat transfer and uniform temperatures throughout the freezing chamber. Dynamic vapor methods only partially relieve the drawbacks of nitrogen gas by circulating the gas through the chamber at a high rate. The metal Cassou rack may be successful, because it conducts most of the heat from straws directly to the liquid nitrogen bath rather than depending on static vapor for cooling. Pellet freezing on dry ice provides fast and efficient heat transfer by using small samples, each with its own cold stream of gas, which at any point in this cooling process is controlled by each sample's temperature. Heat transfer is so efficient that supercooling of a pellet has never been observed. In general, liquid baths (e.g. alcohol) provide convenient, fast, repeatable, and efficient heat transfer for cooling semen, although high cooling rates are restricted to static liquid baths due to the high heat capacity of the liquid.

The Assay Problem

Although correlation to fertility should be the ultimate test of any assay, such correlation is difficult because fertility data have large variability. Also, different assays are possibly more sensitive to different types of damage that are of equal importance to fertility. We studied the sensitivity of a number of laboratory assays