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Storage of bovine semen in liquid and frozen state

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Abstract

This review describes the historical and current methods used for storage of bovine semen. The essential physiological differences between liquid and frozen semen, their relative advantages and disadvantages are addressed, and the current state of technology, the procedures used, their merits and future possibilities are also discussed. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Genetic advancement in the cattle population, particularly in the dairy sector has relied on two processes, namely, the use of bulls of high genetic merit and the selective rearing of calves of high breeding merit as replacements. Artificial insemination has remained the main vehicle for the rapid dissemination of valuable genes and the method of choice for dairy farmers worldwide to improve the genetic quality of their stock. This steady level of genetic progress in dairy cattle is primarily due to advances in semen technology. For the purposes of this review, the reference will be mostly with the dairy industry, for this is where technical advancement in semen technology has been captured most successfully (Chupin and Schuh, 1993; Chupin and Thibier, 1995; Cunningham, 1998; Foote, 1998).

The potential genetic contribution of a sire is described by Foote (1998) as:

$$\text{Contribution of a sire to genetic improvement} = \text{Number of progeny per sire} \times \text{Genetic superiority of the sire}.$$

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The number of progeny per sire will be determined by: (i) total sperm output of the bull; (ii) the number of sperm used per insemination; and (iii) the percentage of cows calving to a single insemination. This can be represented as:

$$\begin{aligned}&\text{Number of progeny per sire}\\&\quad = (\text{Number of sperm per sire}/\text{number of sperm inseminated per cow})\\&\qquad \times \text{Proportion of cows calving to a single insemination.}\end{aligned}$$

These principles effectively determine the number of bulls required for a dairy cow population. The equation becomes particularly attractive when very few bulls are needed to service a large population of dairy cows thereby significantly raising the selection intensity (Shannon, 1978).

The requirement of semen from bulls of high genetic merit has been the main impetus for developing and refining storage technologies for cattle semen. In a 1980 survey, it was believed that the total number of inseminations worldwide exceeded 130 million (Bonadonna and Succi, 1980). A more recent survey (Chupin and Thibier, 1995) showed that the total number of doses of semen produced exceeded 200 million, with more than 95% of this processed as frozen product. About 4 million doses were used as liquid semen, and this was restricted primarily to New Zealand with smaller amounts in France, The Netherlands, Australia and Eastern Europe. In this review, the main focus will be on the well-known storage technologies, which include liquid and frozen stored semen along with a brief discussion on emerging technologies for semen storage in cattle.

2. Liquid storage of semen

There have been many reports since the turn of the century on diluting media for semen of livestock with much of this work originating in the former Soviet Union (Anderson, 1945). Each diluent, ranging from a simple salt solution to the more complex buffered medium had its own merits. Perhaps the accepted principle of semen dilution technology was that survival of spermatozoa for extended periods was inversely related to their metabolic activity. To be useful for artificial insemination, diluted semen had to have a minimum shelf-life of between 2 and 4 days to provide for easy transport and use in distant locations. It was this guiding principle that led to the initial storage temperature of 5°C (Salisbury and Van Demark, 1961). The predominant effect of storage at 5°C was a lowering of metabolic rate of spermatozoa, which contributed to extended survival. The discovery that egg yolk was a useful additive in increasing the preserving properties of the various media, added further impetus to this work (Phillips, 1939). Many extenders have been developed for liquid storage of semen, and this chapter only provides a brief description of some of the major developments. Detailed discussions on some of the early diluents are available in a comprehensive review by Foote (1978).

2.1. *Diluents for storage of semen at refrigerated temperatures*

The early diluents for storage at refrigerated temperatures were significantly influenced by the discovery of egg yolk as a useful additive, and the primary buffering

Table 1
Comparison of 60- to 90-day non-returns (% NRR) to inseminations with bovine semen diluted to 8 million spermatozoa/ml in CUE and Tris media. Data from Foote (1978). Diluent compositions are shown in Table 2

Diluent	No. 1st inseminations	% NRR
CUE	5981	73.0
Tris	5673	73.3

component in these diluents was phosphate (Phillips, 1939). Subsequently, citrate was found to have adequate buffering capacity with an enhanced period of survival of spermatozoa stored at 5°C (Willett and Salisbury, 1942). Citrate then became the salt of choice, as its chelating properties improved the solubility of protein fractions in egg yolk. Egg yolk has been a more common additive, but homogenised whole milk, fresh or reconstituted skim milk and coconut milk have also been used to preserve fertility of bovine spermatozoa (Melrose, 1962; Norman et al., 1962).

Many of the zwitterionic buffers (Good et al., 1966) provided good buffering capacity over a wide pH range. Tris, TES, MES, HEPES, PIPES, MOPS and BES titrated with hydrochloric acid were tested at various pH levels as semen diluents and all of them were similar in suitability (Foote, 1972) cited by Foote (1978). However, the Tris-based diluent has become more universal, and this buffering medium combined with egg yolk and glycerol has been tested extensively (Davis et al., 1963a,b). Motility of spermatozoa was slightly lower in Tris medium buffered to lower pH (6.25 vs. 6.75) than in Cornell University Extender (CUE), but good results were obtained in a fertility trial with both media (Table 1).

A further field trial demonstrated that a lower pH (6.5) and the presence of glycerol in Tris medium improved fertility, compared with higher pH and absence of glycerol (Foote, 1970). The results were comparable with semen diluted with CUE. The conclusion from these series of studies was that the Tris diluent was suitable for storage of semen at refrigerated or ambient temperature and also in frozen state. The diluents shown in Table 2, and some subsequent modifications to these have been used in field trials with varying success (Bartlett and Van Demark, 1962; Van Demark and Bartlett, 1962; Shannon, 1965, 1978; Foote, 1978). In the case of the Illini Variable Temperature (IVT) diluent, the main changes have been in the ratio of bicarbonate to citrate and the concentration of glucose increasing from 0.3% to 1.2%.

2.1.1. Milk-based diluents

The historical origin of milk as a diluent for bull semen has been described by Salisbury and Van Demark (1961). The first report on the use of milk originated in Germany (Koelliker, 1856) cited by Salisbury and Van Demark (1961). This observation went unnoticed until Underbjerg et al. (1942) compared fertility of bull semen stored in egg yolk–phosphate and autoclaved milk diluents. The results were similar for both media. The methods of preparation of milk diluents and the various combinations have been reviewed by Melrose (1962) and Foote (1978). The important aspect of storage of semen in milk-based extenders was the necessity to inactivate the lactenin (a toxic

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Table 2
Composition of diluents for storage of bovine semen at low (5°C) and ambient temperatures. Ingredients are in g/100 ml of medium

Ingredients	Egg yolk–phosphate (Phillips, 1939)	Egg yolk–citrate (Salisbury et al., 1941)	Original IVT diluent (Van Demark et al., 1957)	CUE (Foote et al., 1960)	Tris medium for ambient storage (Foote, 1970)	CAPROGEN® (Shannon, 1965)
Temperature of storage	5°C	5°C	5°C	5°C and ambient	5°C and ambient	Ambient
Tris					3.028	
Potassium hydrogen phosphate	0.2					
Sodium hydrogen phosphate	2					
Sodium citrate		3.6	2	1.45		2
Sodium bicarbonate			0.21	0.21		
Potassium chloride			0.04	0.04		
Glucose			0.3	0.3	1.25	0.3
Citric acid				0.087	1.675	1
Glycine				20		0.014
Glycerol					8	1.25
Catalase						0.003
Caproic acid						0.025
Egg yolk (%)	50	50	10	20	25	5
Antibiotics			Yes	Yes	Yes	Yes
Gas phase			CO_2	self carbonating		N_2

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factor) by heating. Different preparations of milk needed different heating requirements to selectively inactivate the lactenin, but still maintain the integrity of the protein and sugar moieties in milk. Glycerol added to the milk-based diluents was advantageous and maintained fertility of bull spermatozoa for 4 days (O'Connor and Smith, 1959; Almquist, 1962). In general, milk-based extenders have been successful in maintaining fertility and gave comparable results to egg yolk–citrate diluents (Foote, 1978). Egg yolk (5–10%) added to milk-based extenders seemed to inactivate the toxic factor in milk. This process has overcome the problem of heating the skim or fortified milk before addition to semen, and also adequately maintained the fertility of diluted bovine semen stored at 5°C.

2.2. Storage of semen at ambient temperature

It has been the maxim of semen dilution technology that survival of spermatozoa in diluted state is inversely related to their metabolic activity. The pursuit of this principle led to the development of egg yolk diluents for storage of semen at 5°C (Phillips, 1939) and deep freeze techniques for total immobilisation of spermatozoa at very low temperatures (Polge et al., 1949; Polge and Rowson, 1952). In order to store semen at temperatures above 5°C and achieve satisfactory results, alternative methods of metabolic inhibition were attempted. Thus, Van Demark and Sharma (1957), proposed CO_2 narcosis, Norman et al. (1958) suggested lowering the pH and Shannon (1965) proposed N_2 gassing as methods to inhibit metabolic activity of spermatozoa.

Storage at 5°C reduces metabolic activity, but not all changes associated with lower temperatures are beneficial to spermatozoa. For example, the activity of the Na^+/K^+ pump decreases with reduced temperatures such as 5°C, but is unable to cope with diffusion of ions across the cell membrane (Quinn and White, 1966, 1967; Sweadner and Goldin, 1980). A consequent increase in the intracellular concentration of Na^+ is detrimental to survival of spermatozoa (Makler et al., 1981). It was then postulated that storage at ambient temperature may be superior to storage at 5°C, provided the medium in which spermatozoa are suspended inhibits those pathways that are detrimental to their survival at higher temperatures (Shannon and Curson, 1972b, 1982a, 1984). The temperature of storage is an important consideration. The optimum temperature range is considered to be 18°C to 24°C (Shannon and Curson, 1984). Storage at temperatures above this results in lower fertility, compared to storage at 5°C (Foote and Bratton, 1960; Bartlett and Van Demark, 1962).

Another significant development in ambient temperature diluent technology has been the change in the level of egg yolk in the diluent. The ratio of egg yolk to buffer became particularly important at higher storage temperatures than it was with storage at 5°C (Foote and Bratton, 1960; Shannon and Curson, 1983). Historically, semen diluents have included anywhere from 12.5% to 50% egg yolk in the medium. Efforts to decrease the egg yolk concentration in the simple phosphate and citrate buffers gave no advantage in survival of spermatozoa or fertility (Foote and Bratton, 1960). Egg yolk does protect the sperm cells from the toxic effects of seminal plasma; however, it also provides substrates (aromatic amino acids such as L-phenylalanine) for H_2O_2 production by an aromatic amino acid oxidase (AAAO) released from dead cells to the detriment of live

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Table 3
Percentage of NRRs (49 day) for semen diluted in CAPROGEN® media containing differing levels of egg yolk. Data from Shannon and Curson (1983)

Trial	Egg yolk (%)			
	20	5	2	1
1	66.5 ± 0.4	67.6 ± 0.4		
2		67.4 ± 0.3	67.9 ± 0.3	
3		67.6 ± 0.7	67.4 ± 0.7	66.1 ± 0.7

spermatozoa (Shannon and Curson, 1972b, 1982b). The amount of egg yolk required in semen diluents to provide protection against seminal plasma toxins is proportional to the amount of seminal plasma in diluted semen (Shannon and Curson, 1972a). Thus, when semen is diluted to a high rate, and the seminal plasma concentration is consequently reduced, there would be some advantage in reducing the egg yolk concentration. This was borne out in a large fertility trial, where a decrease in egg yolk concentration from 20% to 5% had no detrimental effect on fertility. Further decrease of egg yolk concentration affected fertility of some sires, suggesting that the level of egg yolk was insufficient in some cases to be completely protective (Table 3).

2.2.1. Coconut milk extender

Coconut milk extenders have been equivalent to skim milk–glycerol, CUE or CAPROGEN® in maintaining motility and survival of spermatozoa (Norman and Rao, 1972; Foote, 1978). This extender is quite simple and contains 15% decanted coconut milk boiled for 10 min, 2.2% sodium citrate, antibiotics and 5% egg yolk. The presence of egg yolk was essential to provide a lipid component to the medium. In some cases, the medium has been supplemented with 0.1% calcium carbonate (Norman et al., 1958). A recent report on the use of Coconut extract and Coconut milk on ram semen showed dramatic maintenance of motility over a 48 h period of 30°C but no fertility trials have been reported (Chairussyuhur et al., 1993). There have been no recent reports on significant advances in this front in the literature.

2.2.2. Immobilisation of spermatozoa by low pH

An early observation in 1924 by Krshyshkovsky and Pavlov, cited by Norman et al. (1958), showed that spermatozoa were immobilised when placed in sealed tubes at room temperature, but subsequent exposure to air produced a resumption of activity. Inhibition of sperm motility was due to the decrease of pH by the accumulation of lactic acid in the medium. Further studies by Norman et al. (1958) confirmed the finding and suggested the decrease of pH as an effective method to inhibit metabolic activity of spermatozoa. In this study, conclusive evidence was obtained that lowering the pH substantially reduced metabolic activity measured by O_2 consumption, lactate production, and motility of live spermatozoa. This effect was reversible, as activity resumed when the old diluting medium was replaced after 150 h incubation with fresh medium at neutral pH. By merely altering the pH from 5.76 to 7.45, motile activity of spermatozoa could

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