Short Communication

Preliminary report on a novel straw semen freezing method for ram spermatozoa

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A straw (0.25 ml French) semen freezing method for ram spermatozoa using a temperature controlled stainless steel plate is described to possibly replace the conventional static liquid nitrogen vapour freezing method.

'n Strooitjie (0.25 ml Franse) semenvriesmetode vir ramspermatosoa deur gebruikmaking van 'n temperatuurbeheerde vlekvrye staalplaat word beskryf om moontlik die konvensionele statiese vloeibare stikstofdamp-vriesmetode te vervang.

Keywords: Freezing method, semen straws.

Most results on packaging spermatozoa for cryopreservation are based on freezing of semen in straws (Cassou metal racks) at specific distances (temperatures) above the liquid nitrogen (LN_2) in the static LN_2 vapour.

Freezing curves produced by static vapour 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 (1/1500 of alcohol), a small amount of heat will warm the gas a great deal. The 335 joules of heat from freezing 1 ml of water would warm 1 litre of gas approximately 260 °C. Therefore, static vapour 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 (Graham, 1978).

Freezing straws in LN_2 vapour results in a constantly changing rate of cooling as the internal temperature decreases (Robbins *et al.*, 1976). Cooling rates of 6 to 24 °C/min (Watson & Martin, 1975) and 10 to 100 °C/min (Fiser & Fairfull, 1984) have been reported as acceptable, demonstrating that ram spermatozoa tolerate a wide range of cooling velocities. However, to optimize and standardize freezing, freezing methods subjected to less variation have to be developed.

As ram sperm freezing by the pellet method was 'successfully' accomplished by pelleting onto a pre-cooled stainless steel plate (Salamon, 1970), the objective of this investigation was to develop a straw (0.25 ml French) freezing method making use of a pre-cooled stainless steel plate.

A stainless steel plate (25 × 15 × 1 cm) connected to a thermo couple (Fluke 52, K/J Thermometer) was suspended 4 cm from the bottom in a styrofoam box (ID 21 × 21 × 21 cm plus 6 cm for OD) with lid over LN_2. Using LN_2, the plate was cooled and stabilized at specific sub-zero temperatures.

Semen was collected from two to three S A Mutton Merino rams using an artificial vagina. The ejaculates were pooled and evaluated (undiluted) for sperm concentration (spectrophotometrically) and motility; only samples with a good concentration (>3.0 × 10^9/ml) and motility (>80%) were used. Semen was diluted (30 °C) with a Tris-glucose-citric acid-egg yolk-glycerol diluter (Evans & Maxwell, 1987) to a concentration of 10^9 sperm/ml and packed into 0.25 ml French straws.

Following cooling in a container with water from 30 °C to 4 °C (3 h), the straws (dry) were frozen by placing them directly onto the surface of the stainless steel plate (4 or 8 min) before plunging into LN_2. After storage for at least 24 h the straws were thawed (2/observation) at 50 °C for 10 sec and evaluated for percentage motility (subjectively) and percentage sperm plasma membrane intact cells (Nigrosin/Eosin; Dott & Foster, 1972) at 0 h (30 °C) and 2 or 4 h incubation at 37 °C. For evaluation, thawed sperm were diluted 10 fold with Dulbecco's phosphate buffered saline solution (pH 7.2). These 2 or 4 h recovery values are a more valid indication of post thaw sperm quality and fertilizing potential (Colas, 1975).

Results obtained with the above-mentioned protocol are presented in Tables 1 and 2.

The results (Table 1; motility) compare favourable to that of the solid CO_2 (dry ice) pellet method (Salamon, 1970), with –90 °C appearing to be the optimum plate freezing temperature for ram spermatozoa. For freezing temperatures of –85 °C, –90 °C or –95 °C (Table 1; 10 × 0.25 ml straws each) the plate temperature increased to a high after

Table 1 Survival of ram spermatozoa following steel plate freezing (4 min; mean ± s.e.m.; n = 3)

Plate temperature (–°C)	% Motility		% Sperm plasma membrane intact	
	0 h	4 h	0 h	4 h
80	24.3		29.3	
	±0.7		±3.4	
90	31.7		36.1	
	±1.7		±6.0	
100	26.0		30.8	
	±4.6		±5.2	
120	22.3		24.8	
	±1.5		±1.6	
85	28.3	21.0	42.5	23.9
	±4.4	±3.6	±1.0	±1.1
90	34.3	21.0	44.5	24.5
	±5.4	±9.6	±6.1	±4.9
95	21.7	12.7	32.2	16.8
	±3.3	±4.6	±4.6	±1.7

0 h = Nil hours after thawing (30 °C)
4 h = Four hours incubation at 37 °C after thawing

Table 2 Effect of steel plate (–90°C) freezing period on ram sperm survival (mean ± s.e.m.; n = 4)

Freezing period (min)	% Motility		% Sperm plasma membrane intact	
	0 h	2 h	0 h	2 h
4	22.8	19.0	24.0	12.0
	±3.7	±3.5	±1.2	±1.5
8	18.0	11.5	17.1	7.8
	±3.3	±2.7	±3.2	±2.3

0 h = Nil hours after thawing (30 °C)
2 h = Two hours incubation at 37 °C after thawing

1.5 min freezing (maximum 1.5 °C deviation), whereafter it steadily decreased to just less than 1.0 °C warmer than the starting values after 4 min freezing.

In comparison with previous experience on freezing in LN_2 vapour (same semen processing protocol), steel plate freezing distinguishes itself with a characteristic higher level of sperm vigour and better progressiveness (fast line movement) of sperm.

According to results in Table 2, a 4 min steel plate freezing (–90 °C) period appears to be more advantageous than an 8 min period.

Although the ultimate test of sperm cryopreservation lies in the sperm fertilizing ability (underway for described protocol), it is believed that, with further optimization, steel plate straw semen freezing can be developed into a more standardized (less variable) freezing method than LN_2 vapour freezing. This freezing method can also be made applicable to bull sperm (and other mammalian sperm) cryopreservation.

Advantages of the steel plate freezing method are:

- nearly precise temperature control;
- relative good sperm survival and vigour;
- ease of execution (will be applicable under field conditions);
- cost effective freezing apparatus; and
- low LN_2 consumption (less than 1*l*/freeze)

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